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(54) Abstract Title

Heteroduplex analysis

(57) Synthetic nucleotide constructs for analysis of the human coagulation factor V gene, the human methylenetetrahydrofolate reductase gene, the human prothrombin gene, the human coagulation factor 8 gene and the human von Willebrand gene are disclosed wherein each of said constructs:

- Is capable of forming a duplex with a fragment of its respective gene, whether said gene does or does not carry a particular polymorphism, such that, upon formation of the duplex, the construct spans the point of the possible polymorphism.

- has a nucleotide sequence of up to 500 base-pairs.

- has an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

Also disclosed are methods for examining said genes with said constructs, methods of forming said constructs, test-kits and a method of simultaneously examining a plurality of regions of genomic DNA.

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

This print takes account of replacement documents submitted after the date of filing to enable the application to comply with the formal requirements of the Patents Rules 1995

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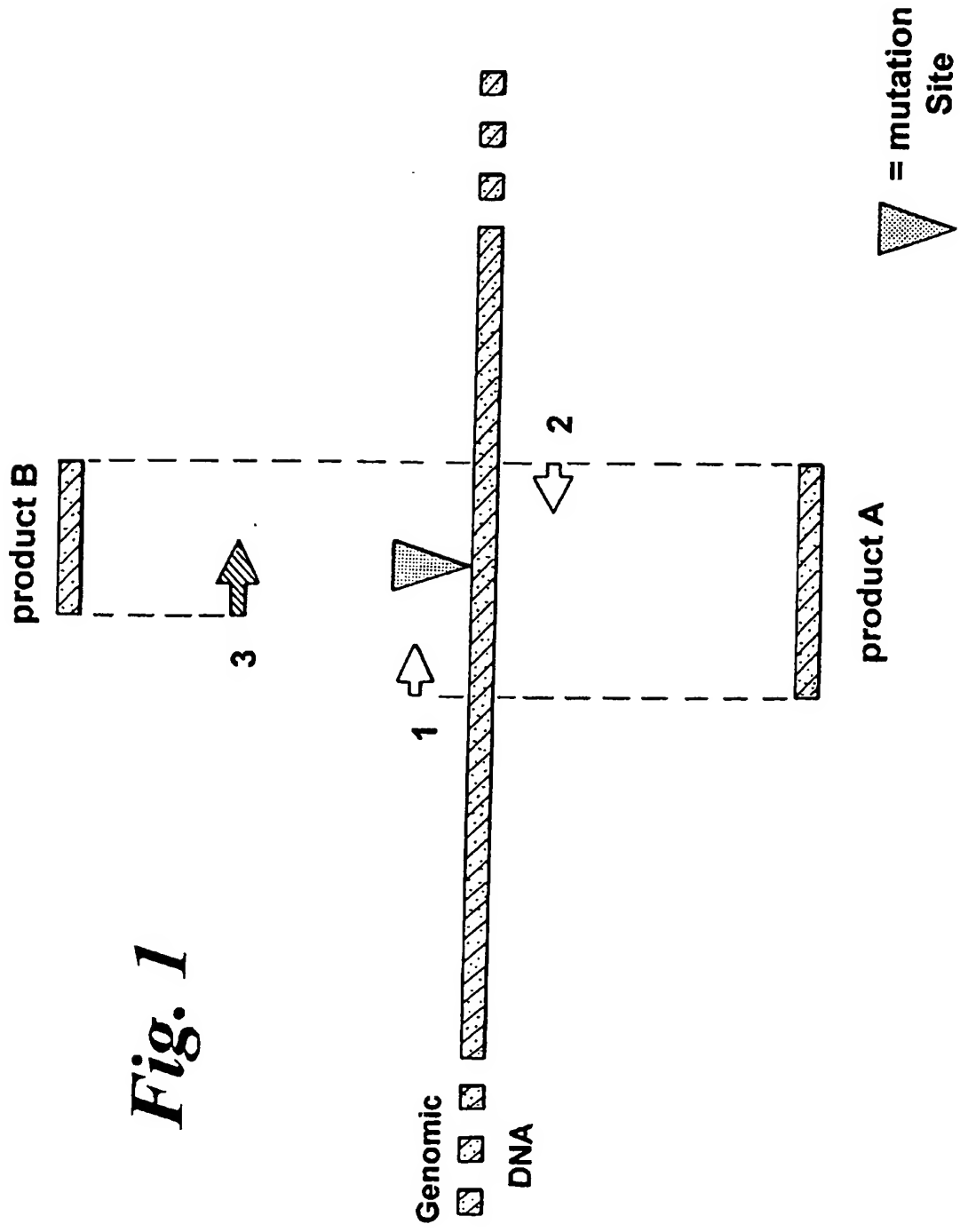


Fig. 1

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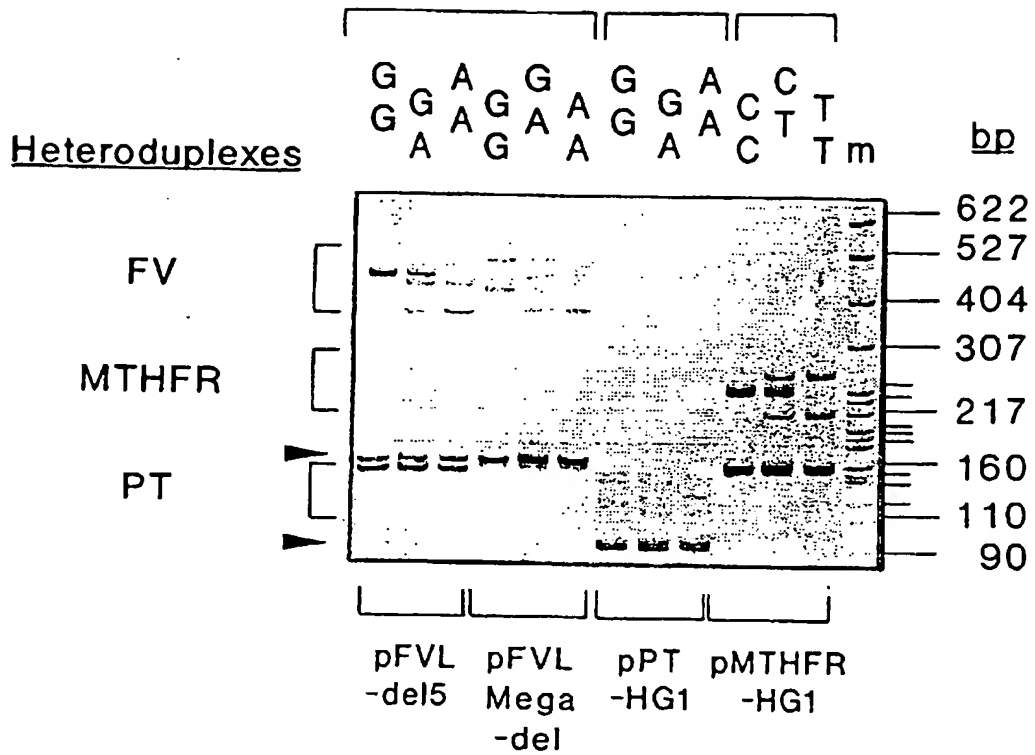


Fig. 2

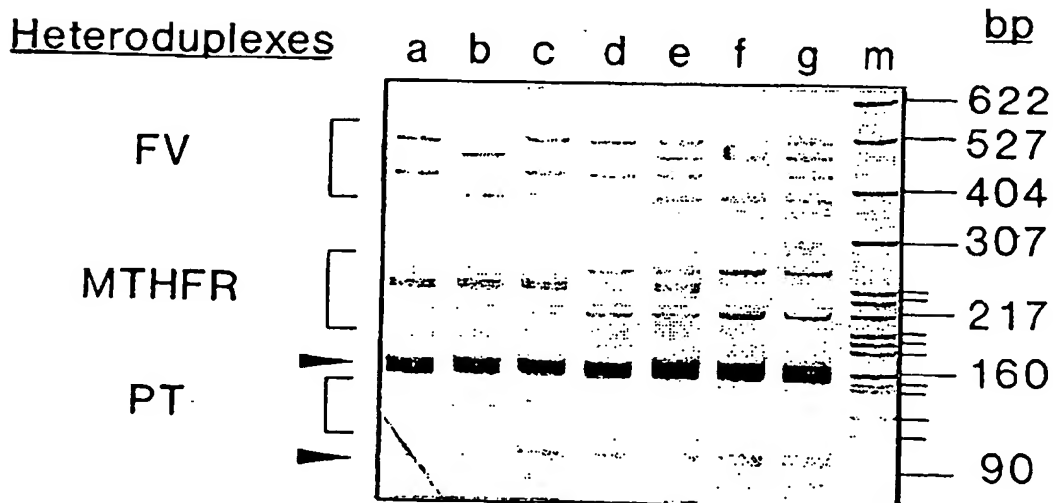
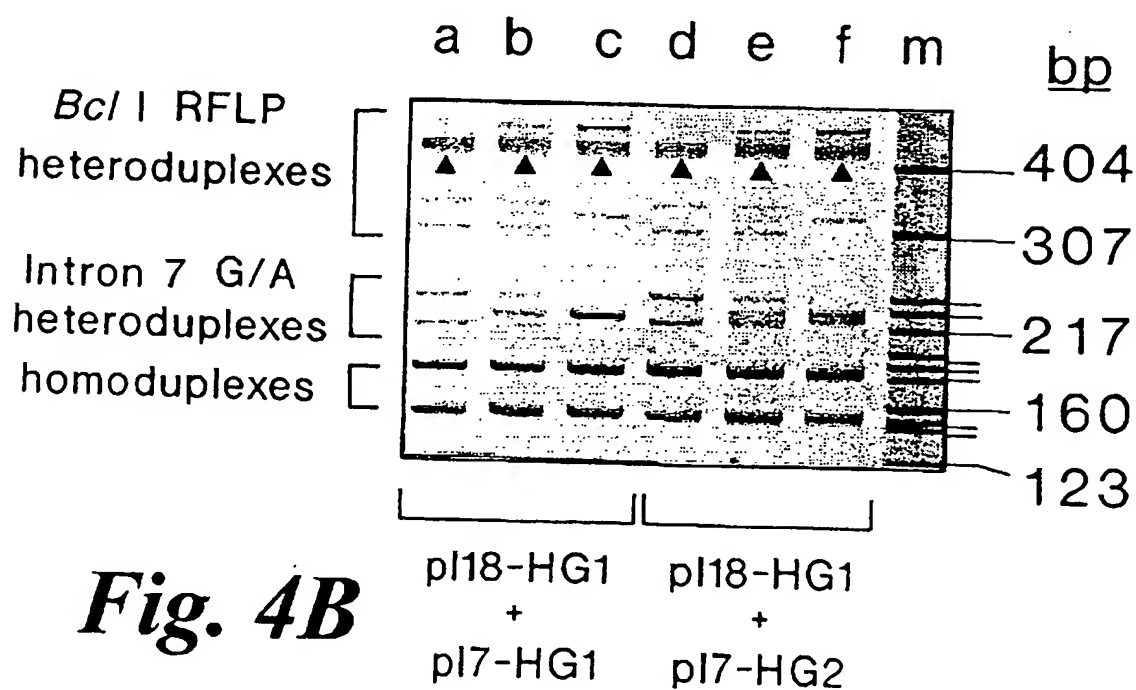
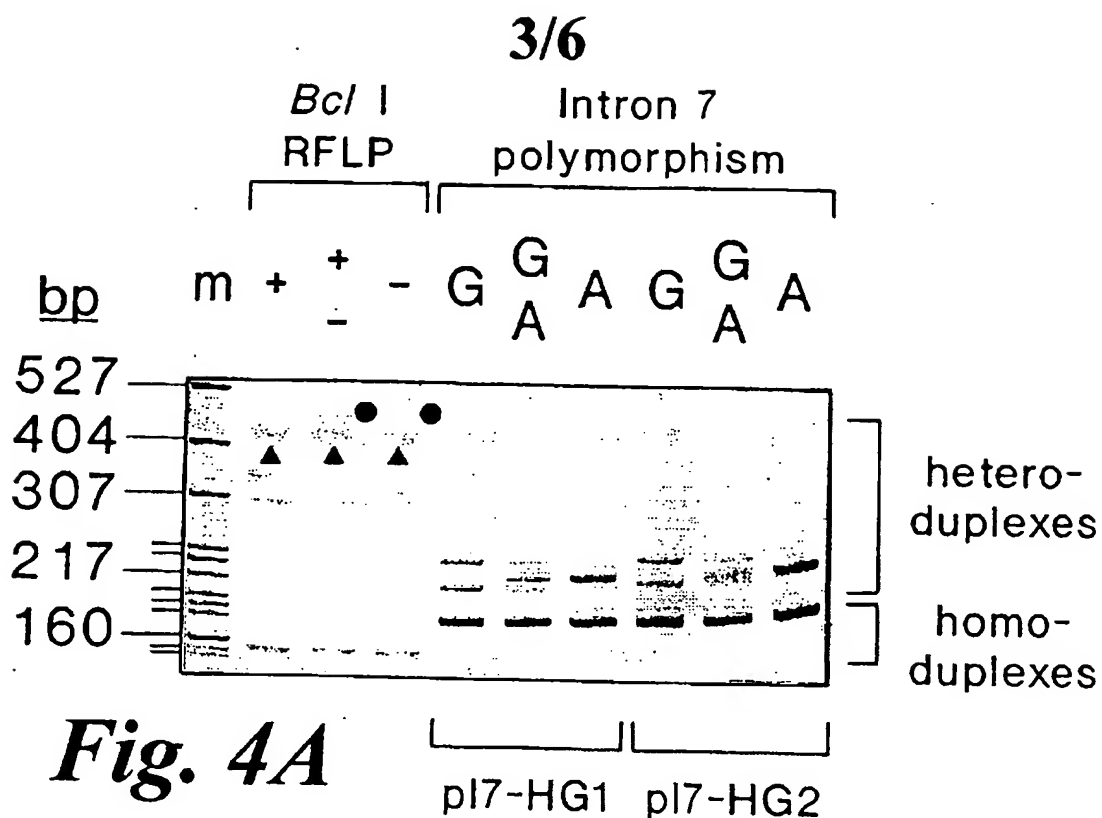


Fig. 3



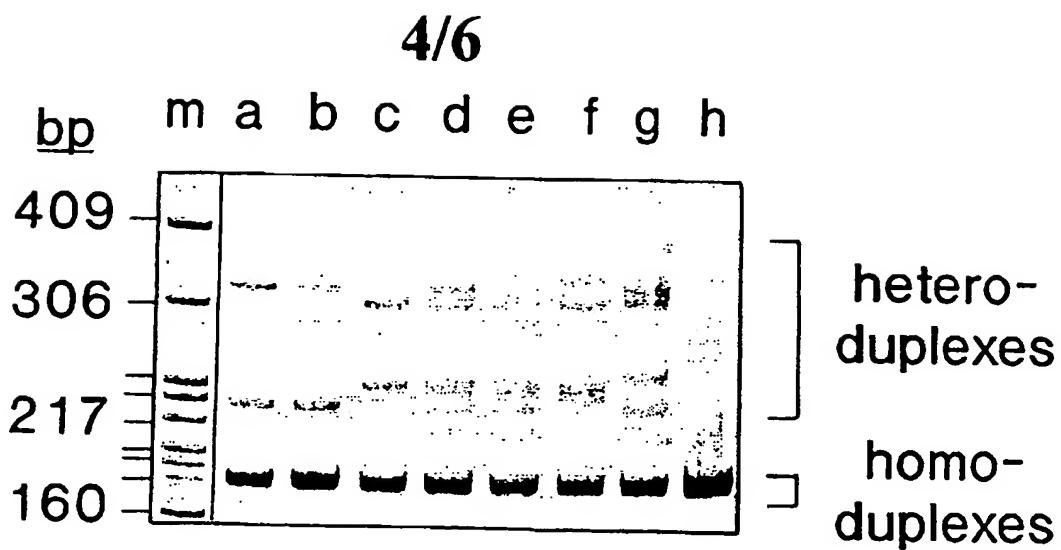


Fig. 5A

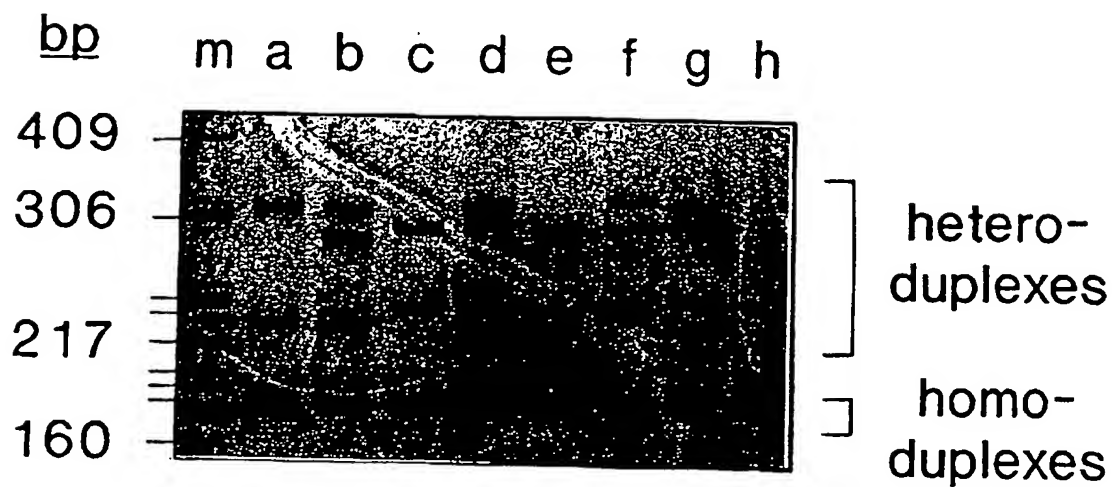


Fig. 5B

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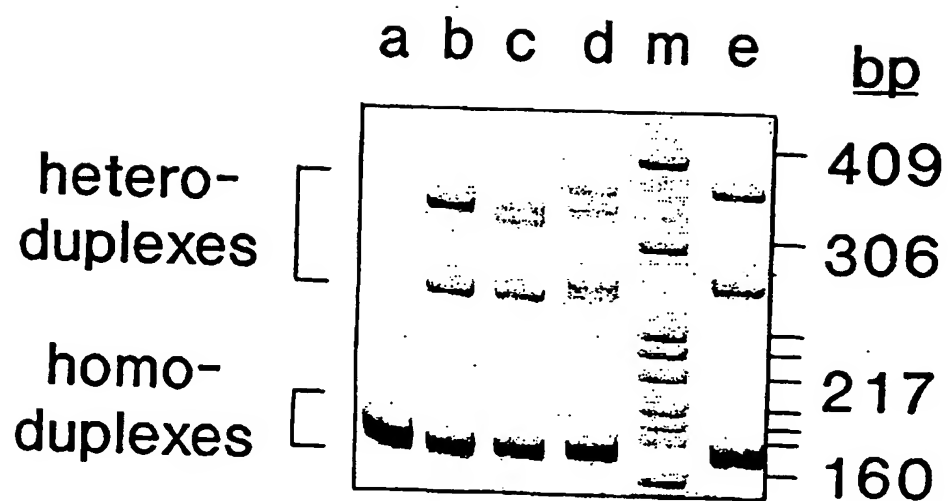
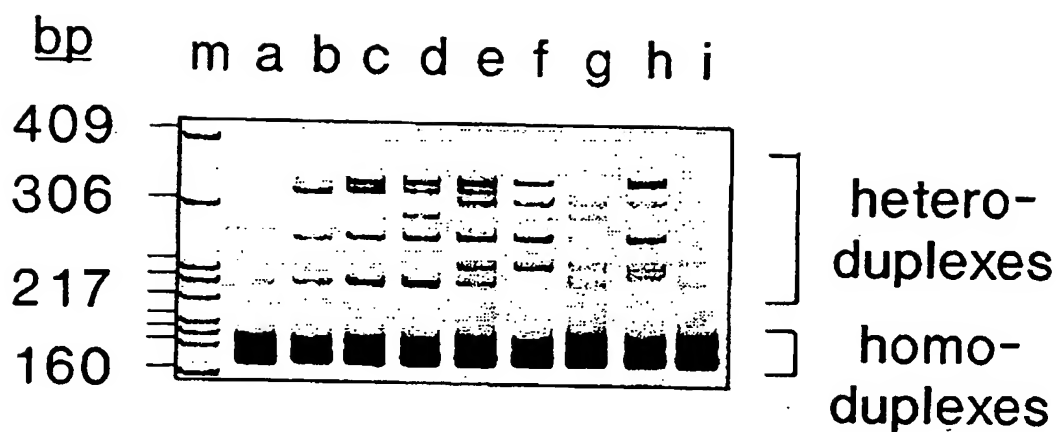
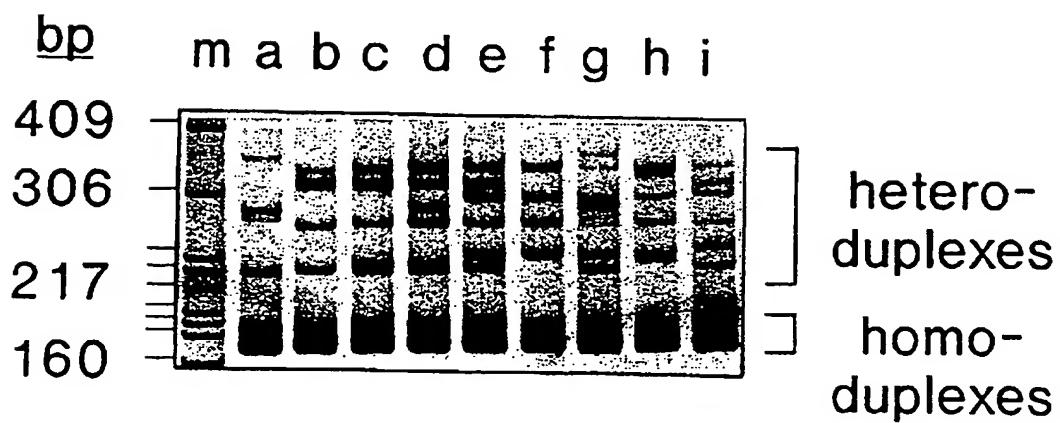


Fig. 6

*Fig. 7A**Fig. 7B*

Heteroduplex Analysis

This invention relates to synthetic nucleotide constructs for examining various genes and a method for examining the genes for mutations or the like. The invention also relates to a method of forming the synthetic constructs which may be used for examining the presence of a mutation in various genes, and a test kit including the synthetic constructs.

Synthetic DNA constructs of the present invention permit rapid genetic diagnosis of certain haemostatically relevant mutations or, for example, permit rapid genotyping of non-pathological nucleotide variants in the human factor 8 gene which are useful in the provision of genetic information for genetic counselling in Haemophilia A (ie. they may simplify and facilitate linkage analysis).

The use of a heteroduplex generator (HG) in heteroduplex analysis is known (WO 93/19201 and Electrophoresis 17: 247-254 (1996)). Heteroduplex generators are synthetic DNA sequences homologous to a genomic DNA sequence but containing deliberate nucleotide deletions, insertions or substitutions at positions vicinal to a known mutation site within that genomic sequence. The HG is designed such that it can be amplified using the same PCR primers as the target sequence in the genomic DNA. Following PCR amplification, the products from genomic DNA and from the HG are mixed together, denatured using heat and rehybridised by slow cooling. This generates DNA heteroduplexes in which one PCR strand is genomic and the other is HG. Dependent upon

whether the genomic strand contains the normal or the mutant sequence, the conformation of the heteroduplex will differ, resulting in different and characteristic electrophoretic mobilities of the two forms. The deliberate deletion/insertion/substitution in the HG is known as an "amplifier" or "identifier": it exaggerates the conformational difference between "normal + HG" heteroduplexes and "mutant + HG" heteroduplexes.

Whilst computer modelling may be used to try to predict the best identifier to use against a given mutation in a given sequence, what is predicted in theory is often not accurate.

In the design of a HG, the following choices are open to the researcher:

- i. The size of the PCR target region in genomic DNA.
- ii. The positions of both primer sequences for genomic amplification.
- iii. The placement of the 5' and 3' extremes of the PCR target region relative to the mutation site.
- iv. The nature of the identifier to be introduced into the HG (deletion/insertion/substitution).
- v. The placement of the identifier relative to the mutation site (5' or 3', immediately adjacent or one or more nucleotides away, or overlapping).
- vi. The size of the identifier (eg 3 nucleotides, 4 nucleotides, 5 nucleotides, etc).
- vii. The inclusion of either the normal nucleotide or the mutant (but not both) at the position within the HG corresponding to the mutation site in the genomic sequence.

There is a need to provide specific synthetic nucleotide constructs for examining different genes.

Once the genomic target region has been decided upon, and the PCR primers designed for its amplification, the HG is synthesised. In the past, one approach used for the synthesis utilises a series of long oligonucleotides (longmers) which overlap such that the 3' end of one can hybridise to the 3' end of a second. A polymerase is then used to "fill in" the outlying, single stranded 5' ends, thereby creating a double stranded fragment which may be the completed HG or which may require further rounds of hybridisation plus "fill-in" with additional longmers until the desired construct is obtained. However, it has been found that the length of the longmer is restricted since yield and fidelity of the sequence decreases with increasing oligonucleotide size. Furthermore, the cost of the HG synthesis increases as the length and the number of longmers needed for its synthesis increases and the possibility of in vitro mutations occurring increases as the number of rounds in the synthesis increases.

A different approach to heteroduplex generator synthesis has been proposed in order to overcome the above-mentioned problems (Thrombosis and Haemostasis 77: 119-122 (1997)). In this approach, only 3 oligonucleotides of approximately 24 bases in length are required for the synthesis of heteroduplex generators ranging in size from tens of bases (for example 80 bp) to several kilobases (for example 8 kb). However, there is a need to adapt this

method to the synthesis of specific heteroduplex generators.

According to a first aspect of the present invention, there is provided a synthetic nucleotide construct for examining the human coagulation factor V (FV) gene, the synthetic construct being capable of forming a duplex with a fragment of the FV gene, which fragment may contain the FV Leiden mutation, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene.

The FV Leiden mutation occurs in codon 506 of the FV coding sequence and is a G to A transition. This transition converts the triplet CGA (encoding arginine) to CAA (encoding glutamine). The mutation occurs at position 1691 in the FV mRNA and, using standard nomenclature, is represented by G1691A (where G is the normal nucleotide and A is the variant nucleotide).

Preferably, the 5 nucleotide deletion is within 50 nucleotides relative to the site of the mutation in the gene, even more preferably within 20 nucleotides, and is even more preferably within 10 nucleotides.

Whilst the deletion may be on either side of the mutation site, in a preferred embodiment the 5 nucleotide deletion comprises nucleotides -6 to -2 relative to the site of the mutation in the gene. The site of the mutation is

defined as nucleotide zero, and locations with a negative value are 5' to this, and locations with a positive value are 3' to this. Thus, the position of the deletion is relative to the position of the known mutation where such a
5 mutation exists in the fragment.

In a preferred embodiment, the length of the synthetic construct is about 141 base pairs long. The mutation is preferably located at the 69th nucleotide from the 5' end of the 146 base pair genomic product, corresponding to nucleotide 64 in the synthetic construct. This synthetic
10 nucleotide construct is known as FVL-del5 and when cloned is known as pFVL-del5.

According to a further aspect of the present invention, there is provided a clone comprising the synthetic
15 nucleotide construct as mentioned above.

Preferably, the synthetic nucleotide construct in the clone further comprises an inserted nucleotide, preferably a C nucleotide, which may be positioned between nucleotides -7 and -8 in the synthetic nucleotide construct (where
20 position zero corresponds to the nucleotide which may be mutated in the genomic DNA). The construct is then of length 142bp and is known as FVL-Megadel. The clone is known as pFVL Megadel.

According to a further aspect of the present invention, there is provided a method of examining the factor V gene comprising the steps of:
25

- a) forming fragments of the factor V gene, which fragments may contain the factor V Leiden mutation;

- (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of possible mutation in the gene, and wherein the sequence of the synthetic construct is such that there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene;
- (c) permitting duplex formation; and
- (d) separating the duplexes formed according to whether the mutation is present.

The synthetic construct may have the preferred features specified above. Furthermore, a recombinant plasmid isolated from the clone mentioned above may be used in this method, without the necessity to release the synthetic construct from the plasmid, but having been amplified as necessary.

Preferably, prior to step (a) a patient's DNA and a sample containing the synthetic construct may be mixed. A PCR may subsequently be carried out to co-amplify the gene and synthetic construct and this preferably leads to the formation of duplexes between amplified gene fragments and amplified synthetic construct fragments as a result of cyclical denaturation and hybridisation.

The separation of the duplexes is preferably carried out by electrophoresis, for example gel electrophoresis.

According to a further aspect of the present invention, there is provided a method of forming a synthetic nucleotide

construct for examining the human coagulation factor V gene, which may contain the factor V Leiden mutation, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of a duplex, and wherein the sequence of the synthetic construct is such that there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene, the method comprising the steps of:

- (a) generating a first fragment of the factor V gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain the mutation;
- (b) generating, from the gene or from the first fragment, a second fragment containing the 5 nucleotide deletion using the first or second primer and a third primer which contains the deletion;
- (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and
- (e) amplifying the extended strand.

The extended strand is preferably amplified to produce a double stranded product. The method may further comprise the step of isolating the extended strand or its double stranded amplification product and this may be carried out by electrophoresis. The third primer may be designed to pair with either the second primer (in which case it is a "sense" primer), or to pair with the first primer (in which

case it is an "anti-sense" primer). The principle of synthesis is identical, irrespective of which of these choices is made. Thus, in step (b) the third primer generates the second fragment by pairing with either the first or second primer. Preferably, the part of the nucleotide sequence which may contain the mutation is between the third primer and whichever of the first or second primer is used, although it has been found that the mutation site may or may not be between the third primer and whichever of the first or second primer is used.

The method may further comprise the step of cloning the synthetic construct into a plasmid vector.

Preferably, the third primer is

5'-AAGAGCAGATCCCTGGCGAGGAATACAGGTACTT-3' [SEQ ID No:1], known as FvL:3. In a preferred embodiment the first primer is 5'-CATGAGAGACATCGCCTCTG-3' [SEQ ID No:2], known as FvL:1 and the second primer is independently

5'-GACCTAACATGTTCTAGCCAGAAG-3' [SEQ ID No:3], known as FvL:2. However, any suitable primers may be used. However, if the size of the genomic PCR product is to be kept the same and the position of the mutation within it is to be the same, the 5' ends of primers 1 and 2 are fixed but the 3' ends could be varied, for example to give primers of length 15 to 30 nucleotides. Preferably, for the third primer, the 5' end lies immediately downstream of a T in the sense strand or A in the anti-sense strand. Whilst there should be sufficient sequence either side of the deletion to "clamp" the primer down, the length of the sequence either side can

vary within this constraint.

According to a further aspect of the present invention, there is provided a test kit for examining the human coagulation factor V gene, the kit comprising:

- 5 (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence
10 under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the factor V Leiden mutation is present within the sequence under examination;
- 15 and
- (c) a control DNA and/or control PCR amplification product, wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein
20 the sequence of the synthetic construct is such that there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene.

In the present case, the synthetic construct is preferably FVL-Megadel, for example in its cloned plasmid
25 form.

According to a further aspect of the present invention, there is provided a synthetic nucleotide construct for examining the human methylenetetrahydrofolate reductase

(MTHFR) gene, the synthetic construct being capable of forming a duplex with a fragment of the MTHFR gene, which fragment may contain a C to T mutation at nucleotide 677 in the coding sequence of the mRNA, wherein the nucleotide
5 sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible
10 mutation in the gene.

Preferably, the identifier is within 50 nucleotides relative to the site of the possible mutation in the gene, even more preferably within 20 nucleotides, and even more preferably within 10 nucleotides.

15 Whilst the identifier may be on either side of the mutation site, in a preferred embodiment, the identifier is a 3 nucleotide deletion, which may comprise nucleotides -4 to -2 relative to the site of the mutation in the gene.

The MTHFR gene fragment is preferably of a length of
20 147 bp and the 5' and 3' extremes are preferably positioned such that the mutation site may reside 54 nucleotides downstream relative to the 5' end.

The synthetic construct has been found to provide a very clear distinction between normal and mutant nucleotides
25 on heteroduplex analysis and, for example, the three genotypes C+C, C+T and T+T were easily distinguishable. The synthetic construct is known as MTHFR-HG1.

According to a further aspect of the present invention,

there is provided a clone comprising the above-mentioned synthetic nucleotide construct.

A specific clone is known as pMTHFR-HG1.

- According to a further aspect of the present invention,
- 5 there is provided a method of examining the MTHFR gene, comprising the steps of:
- (a) forming fragments of the MTHFR gene, which fragments may contain a C to T mutation at nucleotide 677 in the coding sequence of the mRNA;
 - 10 (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene,
 - 15 and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene;
 - (c) permitting duplex formation; and
 - (d) separating the duplexes formed according to whether the
 - 20 mutation is present.

The synthetic construct may have the preferred features specified above.

As mentioned above, in relation to the method for examining the FV gene, prior to step (a) the DNA of the

25 patient and the sample containing the synthetic construct may be mixed. A PCR may subsequently be carried out to co-amplify the gene and synthetic construct and this preferably leads to the formation of duplexes between amplified gene

fragments and amplified synthetic construct fragments as a result of cyclical denaturation and hybridisation. A recombinant plasmid isolated from a clone comprising the synthetic nucleotide construct can be used directly in the method, without the necessity to release the synthetic construct from the plasmid. Again, in step d, the separation is preferably by electrophoresis.

According to a further aspect of the present invention, there is provided a method of forming a synthetic construct for examining the human MTHFR gene which may contain a C to T mutation at nucleotide 677 in the coding sequence of mRNA, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene, the method comprising the steps of:

- (a) generating a first fragment of the MTHFR gene by a first and a second primer on either side of the part of the nucleotide sequence which may contain the mutation;
- (b) generating from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;
- (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and

(e) amplifying the extended strand.

As above, the extended strand is preferably amplified to produce a double stranded product. The method may further comprise the step of isolating the extended strand or its double-stranded amplification product, preferably by electrophoresis. In addition, the third primer may be "sense" primer or "anti-sense" primer as defined above. When the third primer pairs with either the first or the second primer (whichever is used), they may be on either side of the part of the nucleotide sequence which may contain the mutation, although this is not essential.

The method may further comprise the step of cloning the extended strand in to a plasmid vector.

Preferably, the third primer has a nucleotide sequence of 5'-GAAGGAGAAGGTGTCTGCGCCGATTTTCATCATCA-3' [SEQ ID No:4], known as MTHFR:3 and the first and second primers preferably independently have the sequence 5'-AGGGAGCTTTGAGGCTGACCTGAA-3' [SEQ ID No:5], known as MTHFR:1 and 5'-ACGATGGGGCAAGTGATGCCCATG-3' [SEQ ID No:6], known as MTHFR:2 respectively. However, any suitable primers can be used.

According to a further aspect of the present invention, there is provided a test kit for examining the human MTHFR gene, the kit comprising:

- (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is

capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the C to T mutation at nucleotide 677 in the coding sequence of the mRNA is present within the sequence under examination; and

(c) a control DNA and/or control amplification product, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene.

In the present case the synthetic construct is preferably pMTHFR-HG1, for example in its cloned, plasmid form.

According to a further aspect of the present invention, there is provided a synthetic nucleotide construct for examining the gene encoding human prothrombin, the synthetic construct being capable of forming a duplex with a fragment of the gene encoding prothrombin, which fragment may contain a G to A mutation at nucleotide 20210 in the gene sequence, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative

to the site of the possible mutation in the gene.

The identifier is preferably within 50 nucleotides relative to the site of the mutation in the gene, even more preferably within 20 nucleotides, and even more preferably
5 within 10 nucleotides.

The identifier may be on either side of the mutation site. Preferably, the identifier is a 3 nucleotide deletion, and may comprise nucleotides +1 to +3 relative to the site of the mutation.

10 The synthetic construct preferably has a length of 82 base pairs. The synthetic construct is known as PT HG-3'.

The fragment of the gene is preferably 85 base pairs in length and the mutation, where it exists, may be 43 nucleotides downstream of the 5' end.

15 According to a further aspect of the present invention, there is provided a clone comprising a synthetic nucleotide construct as mentioned above.

A particular clone is known as pPT-HG1.

20 According to a further aspect of the present invention, there is provided a method of examining the gene encoding human prothrombin comprising the steps of:

- (a) forming fragments of the human prothrombin gene, which fragments may contain a G to A mutation at nucleotide 20210 in the gene;
- 25 (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length

and spans the point of the possible mutation in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene;

- 5 (c) permitting duplex formation; and
- (d) separating the duplexes formed according to whether the mutation is present.

The synthetic construct may have the preferred features set out above.

- 10 As mentioned above, prior to step (a) a patient's DNA and sample containing synthetic construct may be mixed. A PCR may subsequently be carried out to co-amplify the gene and synthetic construct and this preferably leads to the formation of duplexes between amplified gene fragments and
- 15 amplified synthetic construct fragments as a result of cyclical denaturation and hybridisation. A recombinant plasmid isolated from a clone comprising the synthetic nucleotide construct can be used directly in the method, without the necessity to release the synthetic construct
- 20 from the plasmid. The separation is preferably by electrophoresis, for example gel electrophoresis.

- According to a further aspect of the present invention, there is provided a method of forming a synthetic nucleotide construct for examining the gene encoding human prothrombin
- 25 which may contain a G to A mutation at nucleotide 20210 in the gene sequence, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene, and wherein there is an identifier within 100

nucleotides relative to the site of the possible mutation in the gene, the method comprising the steps of:

- (a) generating a first fragment of the gene encoding prothrombin using a first and second primer on either side
5 of the part of the nucleotide sequence which may contain the mutation;
- (b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the
10 identifier;
- (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it
15 to the end thereof; and
- (e) amplifying the extended strand.

Again, the extended strand may be amplified to produce a double stranded product and the method may further comprise the step of isolating the extended strand or its
20 double stranded product, preferably by electrophoresis. In addition, the third primer may be a "sense" primer or an "anti-sense" primer. When the third primer pairs with either the first or the second primer (whichever is used), they may be on either side of the part of the nucleotide
25 sequence which may contain the mutation, although this is not essential.

Preferably the third primer has the sequence
5'-GACTCTCAGCGCTCAATGCTC-3' [SEQ ID No:7], known as PT:3.

The first and second primers may independently have the sequences

5'-GTGTTTCTAAACTATGGTTCCCAA-3' [SEQ ID No:8], known as PT:1
and

5 5'-CCCAGAGAGCTGCCCCATGAATAG-3' [SEQ ID No:9], known as PT:2
respectively. However, any suitable primers may be used.

According to a further aspect of the present invention, there is provided a test kit for examining the gene encoding prothrombin, the kit comprising:

- 10 (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence
15 under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the G to A mutation at nucleotide 20210 is present within the sequence under
20 examination; and
- (c) a control DNA and/or control PCR amplification product, wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein
25 the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene.

According to a further aspect of the present invention,

there is provided a synthetic nucleotide construct for examining the human coagulation factor 8 (F8) gene, the synthetic construct being capable of forming a duplex with a fragment of the F8 gene, which fragment may contain a T to

5 A polymorphism in intron 18 of the F8 gene, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that

10 there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

The identifier is preferably within 50 nucleotides relative to the site of the polymorphism in the gene, even more preferably within 20 nucleotides, and even more

15 preferably within 10 nucleotides.

The identifier may be on either side of the polymorphism. The identifier is preferably a 5 nucleotide deletion, which may comprise nucleotides +2 to +6 relative to the site of the polymorphism in the gene. Preferably,

20 the identifier further comprises an insertion of a nucleotide, preferably an insertion of a T nucleotide, which may be between nucleotides +9 and +10 relative to the site of the polymorphism in the gene (corresponding to nucleotides +4 and +5 in the synthetic construct).

25 Preferably, the synthetic nucleotide construct has a length of 138 base pairs (corresponding to a genomic PCR product of 142 base pairs).

The synthetic nucleotide construct is preferably used

for examining the BclI restriction fragment length polymorphism (RFLP) locus in intron 18 of the F8 gene. The synthetic construct is known as F8 I18HG-1.

According to a further aspect of the present invention,
5 there is provided a clone comprising a synthetic nucleotide construct for examining the F8 gene as mentioned above.

A particular clone is known as pF8 I18HG-1.

According to a further aspect of the present invention,
there is provided a method of examining the human coagulation F8 gene comprising the steps of:
10

- (a) forming fragments of the human coagulation F8 gene, which fragments may contain a T to A polymorphism in intron 18 of the F8 gene;
- (b) combining the fragments with a synthetic nucleotide
15 construct, which synthetic construct is capable of forming a duplex with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene, and wherein the sequence of the synthetic construct is
20 such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene;
- (c) permitting duplex formation; and
- (d) separating the duplexes formed according to whether the
25 polymorphism is present.

The synthetic nucleotide construct may have the preferred features mentioned above. A recombinant plasmid isolated from the clone comprising the synthetic nucleotide

construct may be used directly in the method.

As indicated above, prior to step (a) a patient's DNA may be mixed with a sample containing the synthetic construct. A PCR may subsequently be carried out to co-amplify
5 the gene and synthetic construct and this preferably leads to the formation of duplexes between the amplified gene fragments and amplified synthetic construct fragments as a result of cyclical denaturation and hybridisation. A clone comprising the synthetic nucleotide construct can be used
10 directly in the method, without the necessity to release the synthetic construct from the plasmid. The separation step may be by electrophoresis, for example gel electrophoresis.

According to a further aspect of the present invention,
15 there is provided a method of forming a synthetic nucleotide construct for examining the human coagulation F8 gene which may contain a T to A polymorphism in intron 18 of the F8 gene, which construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on
20 formation of a duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene, the method comprising the steps of:

25 (a) generating a first fragment of the human coagulation F8 gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain the polymorphism;

- (b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;
- 5 (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and
- 10 (e) amplifying the extended strand.

The extended strand is preferably amplified to produce a double stranded product. The method may further comprise the step of isolating the extended strand or its double stranded amplification product and this may be by electrophoresis, for example gel electrophoresis. The isolation of the strand may take place by excision of the relevant area of the gel and diffusion into water. As mentioned above, the third primer may be a sense or anti-sense primer and when it pairs with the first or second primer, the primers may be on either side of the part of the nucleotide sequence which may contain the polymorphism although this is not essential.

It has been found that preferred primers may be as follows. The third primer is preferably

25 5'-CAGTGATCAGATCAAGCTCCATGCTC-3' [SEQ ID No:10], known as F8I18:3 and the first and second primers are independently preferably

5'-TAAAAGCTTTAAATGGTCTAGGC-3' [SEQ ID No:11], known as

F8I18:1

5'-TTCGAATTCTGAAATTATCTTGTTTC-3' [SEQ ID No:12], known as F8I18:2 respectively. However, any suitable primers may be used.

5 According to a further aspect of the present invention, there is provided a test kit for examining the human coagulation F8 gene, the kit comprising:

(a) two primers suitable for use in PCR and capable of annealing two complementary sequences at respective ends of
10 a nucleotide sequence to be examined;

(b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are
15 formed between the construct and the nucleotide sequence under examination dependent upon whether a T to A polymorphism in intron 18 of the F8 gene is present within the sequence under examination; and

(c) a control DNA and/or control PCR amplification product,
20 wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible
25 polymorphism in the gene.

According to a further aspect of the present invention, there is provided a synthetic nucleotide construct for examining the human coagulation F8 gene, the synthetic

construct being capable of forming a duplex with a fragment of the human coagulation F8 gene, which fragment may contain a G to A polymorphism in intron 7, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

10 The identifier is preferably within 50 nucleotides relative to the site of the polymorphism in the gene, and is even more preferably within 20 nucleotides relative to the point of the polymorphism in the gene, even more preferably within 10 nucleotides.

15 The identifier may be on either side of the polymorphism site. In a preferred embodiment, the identifier is an insertion of 3 nucleotides, preferably 3 C nucleotides, and may be positioned between nucleotides -1 and -2 relative to the site of the polymorphism in the genomic PCR product. The identifier preferably further comprises an insertion of a nucleotide, preferably a C nucleotide, between nucleotides +6 and +7 relative to the site of the polymorphism in the genomic PCR product (corresponding to nucleotides +6 and +7 in the synthetic construct).

25 The polymorphism is preferably located at nucleotide 27 downstream of the 5' end of the 173 base pair genomic PCR product (which corresponds to nucleotide 30 in the synthetic

construct).

The length of the synthetic construct is preferably 177 base pairs.

According to a further aspect of the present invention,
5 there is provided a clone containing a synthetic nucleotide construct of the form defined above. A particular clone may be known as pF8 I7HG-2. Preferably, the synthetic nucleotide construct in the clone comprises a substitution, preferably a C to T substitution, which may be positioned at
10 the middle nucleotide of the CCC insert. Such a clone is known as pF8I7HG-1.

According to a further aspect of the present invention, there is provided a method of examining the human coagulation F8 gene comprising the steps of:

- 15 (a) forming fragments of the F8 gene, which fragments may contain the G to A polymorphism in intron 7;
- (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence
20 of the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the
25 gene;
- (c) permitting duplex formation; and
- (d) separating the duplexes formed according to whether the polymorphism is present.

The synthetic construct may have the preferred or alternative features specified above. Furthermore, a recombinant plasmid isolated from the clones mentioned above, preferably pF8I7HG-1, may be used in this method, without the necessity to release the synthetic construct from the plasmid, but having been amplified as necessary.

The method may, as above, comprise the further steps of mixing the patient's DNA and the synthetic construct (which need not be released from the plasmid where appropriate) followed by PCR as mentioned above. The separation is preferably carried out by electrophoresis, for example gel electrophoresis.

According to a further aspect of the present invention, there is provided a method of forming a synthetic nucleotide construct for examining the human coagulation F8 gene, which may contain a G to A polymorphism in intron 7, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of a duplex, and wherein there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene, the method comprising the steps of:

- (a) generating a first fragment of the F8 gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain the polymorphism;
- (b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the

identifier;

(c) mixing the first and second fragments in the presence of the first, second and third primers;

(d) hybridising a strand of the second fragment to a
5 complementary strand of the first fragment and extending it to the end thereof; and

(e) amplifying the extended strand.

The synthetic construct, and the nature of the identifier, may be in the form mentioned above.

10 The extended strand is preferably amplified to produce a double stranded product. As mentioned above, the method may further comprise the step of isolating the extended strand or its double stranded amplification product, for example by electrophoresis. The third primer may also be a
15 "sense" or "anti-sense" primer and may pair with either the first or second primer on either side of the part of the nucleotide sequence which may contain the polymorphism although this is not essential.

Preferably, the sequence of the third primer is
20 5'-AGCAAGACACTCCCCTGACATTGCTTTGG-3' [SEQ ID No:13], known as F8I7:3. The sequence of the first primer is preferably 5'-AACAGCCTAATATAGCAAGACACTC-3' [SEQ ID No:14], known as F8I7:1. The sequence of the second primer is preferably 5'-CACATCCATTTCAGAATCAGTAAG-3' [SEQ ID No:15], known as
25 F8I7:2. However, any suitable primers may be used.

According to a further aspect of the present invention, there is provided a test kit for examining the human coagulation F8 gene, the kit comprising:

(a) two primers suitable for use in PCR and capable of annealing to complementary sequences at the respective ends of a nucleotide sequence to be examined;

(b) a synthetic construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the G to A polymorphism in intron 7 is present within the sequence under examination; and

(c) a control DNA and/or control amplification product, wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

According to a further aspect of the present invention, there is provided a synthetic nucleotide construct for examining the human von Willebrand factor (VWF) gene, the synthetic construct being capable of forming a duplex with a fragment of the VWF gene, which fragment may contain one or more mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the coding sequence of the cDNA, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the

point of at least one of the possible mutations in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of one of the possible mutations in the gene.

Preferably, the identifier is within 50 nucleotides relative to the site of at least one of the mutations in the gene, and more preferably within 20 nucleotides and even more preferably within 10 nucleotides. In a particularly preferred embodiment, the identifier is positioned in a relative position between the site of the two possible mutations mentioned above.

Whilst the deletion may be on either side of the mutation site, in a preferred embodiment the identifier is a 3 nucleotide deletion, which may comprise nucleotides +2 to +4 on the 3' side of the G to A transition (which corresponds to nucleotides -8 to -10 on the 5' side of the G to T transversion).

Preferably, the length of the synthetic construct is 173 bp. It may be synthesised from a genomic product having a length of 176 bp by deletion of nucleotides 49 to 51 of the genomic product.

The genomic DNA may additionally or alternatively contain a polymorphism which is a G to A transition at position 2805. The 3 nucleotide deletion in the synthetic construct then may comprise nucleotides +8 to +10 relative to the polymorphism. Therefore, the synthetic nucleotide construct is capable of examining the presence of any or all

of the 3 sequence variants (transversions or transitions) where they are present.

According to a further aspect of the present invention, there is provided a clone comprising a synthetic nucleotide
5 construct for examining the VWF gene as mentioned above. A particular clone is known as pVWFex20-UHG1.

The synthetic construct may further comprise an A to G transition, preferably at nucleotide 31 in the synthetic construct and/or a T to G transversion at nucleotide 37 in
10 the synthetic construct. The clone may comprise the A to G transition and/or G to T transversion. Such a clone is known as pVWFex20-UHG2.

According to a further aspect of the present invention, there is provided a method of examining the human von
15 Willebrand factor (VWF) gene comprising the steps of:

(a) forming fragments of the VWF gene, which fragments may contain one or more mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the
20 coding sequence of the cDNA;

(b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length
25 and spans the point of at least one of the possible mutations in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one of the

possible mutations in the gene;

(c) permitting duplex formation; and

(d) separating the duplexes formed.

The synthetic constructs may have the features specified above. Furthermore, a recombinant plasmid isolated from the clones may be used in this method, without the necessity to release the synthetic construct from the plasmid, but having been amplified as necessary.

Prior to step (a) a patient's DNA and a sample containing the synthetic construct may be mixed. A PCR may subsequently be carried out to co-amplify the gene and synthetic construct and this preferably leads to the formation of duplexes between amplified gene fragments and amplified synthetic construct fragments as a result of cyclical denaturation and hybridisation.

The separation of the duplexes is preferably carried out by electrophoresis, for example gel electrophoresis.

According to a further aspect of the present invention, there is provided a method of forming a synthetic nucleotide construct for examining the human VWF gene which may contain one or more mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the coding sequence of the cDNA, which construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene, the method

comprising the steps of:

- (a) generating a first fragment of the VWF gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain at least one of the mutations;
5
- (b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;
- 10 (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and
- 15 (e) amplifying the extended strand.

As mentioned above in the methods for forming other synthetic constructs, the method may further comprise the step of extending the strand to produce a double stranded product and may also comprise the step of isolating the
20 extended strand or its double stranded amplification product, preferably by electrophoresis. The third primer may be a sense or anti-sense primer and whichever of the first or second primers it pairs with, they may be positioned on either side of the part of the nucleotide sequence
25 which may contain at least one of the mutations, although this is not essential.

The method may further comprise the step of cloning the synthetic construct into a plasmid vector.

Preferably the third primer is 5'-GTCGGGACCGGTGGAAGTGCAC-3' [SEQ ID No:16], known as VWF Ex20:3. The first primer may be 5'-CAACTTGTCATCTCTGCCATGACTG-3' [SEQ ID No:17], known as VWF Ex20:1 and the second primer may independently be 5'-CTCACCTGCACCAGAACGTACTGG-3' [SEQ ID No:18], known as VWF Ex20:2. However, any suitable primer may be used.

According to a further aspect of the present invention, there is provided a test kit for examining the human VWF gene, the kit comprising:

- (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
 - (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether at least one of the mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the coding sequence of the cDNA is present; and
 - (c) a control DNA and/or control PCR amplification product,
- wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene, and wherein the sequence of the synthetic construct is such that there is an ident-

ifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene.

According to a further aspect of the present invention, there is provided a synthetic nucleotide construct for
5 examining the human VWF gene, which fragment may contain one or more mutations including at least a C to T transition at position 2696 in the coding sequence of the cDNA and/or a T to A transversion at position 2701 in the coding sequence of the cDNA, wherein the nucleotide sequence of the synthetic
10 construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one
15 of the possible mutations in the gene.

Preferably, the identifier is within 50 nucleotides relative to the site of at least one of the possible mutations and even more preferably is within 20 nucleotides, even more preferably within 10 nucleotides.

20 In one embodiment, the identifier is a 3 nucleotide insertion, preferably an insertion of 3 A nucleotides, which may be between nucleotides 0 and +1 relative to the site of the C to T transition (which corresponds to -5 and -4 relative to the T to A transversion).

25 Preferably, the identifier is in a relative position between the site of the two possible mutations.

The length of the synthetic construct is preferably 165 bp and may be synthesised from a genomic product of length

162 bp and the identifier may be located between nucleotides 62 and 63 of the genomic product.

According to a further aspect of the present invention, there is provided a clone comprising a synthetic nucleotide
5 construct for examining the VWF gene as mentioned above. Such a clone may be known as pVWFex19-UHG1.

According to a further aspect of the present invention, there is provided a method of examining the human VWF gene, comprising the steps of:

- 10 (a) forming fragments of the VWF gene, which fragments may contain one or more mutations including at least a C to T transition at position 2696 in the coding sequence of the cDNA and/or a T to A transversion at position 2701 in the coding sequence of the cDNA;
- 15 (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of at least one of the possible muta-
20 tions in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene;
- (c) permitting duplex formation; and
- 25 (d) separating the duplexes formed.

The synthetic constructs may have the preferred features specified above. Furthermore, a recombinant plasmid isolated from a clone may be used in this method,

without the necessity to release the synthetic construct from the plasmid.

As mentioned previously, prior to step (a), a patient's DNA and a sample containing the synthetic construct may be mixed. A PCR may subsequently be carried out to co-amplify the gene and synthetic construct and this preferably leads to the formation of duplexes between amplified gene fragments and amplified synthetic fragments as a result of cyclical denaturation and hybridisation.

The separation of the duplexes is preferably carried out by electrophoresis, for example gel electrophoresis.

According to a further aspect of the present invention, there is provided a method of forming a synthetic nucleotide construct for examining the human VWF gene which may contain one or more mutations including at least a C to T transition at position 2696 in the coding sequence of the cDNA and/or a T to A transversion at position 2701 in the coding sequence of the cDNA, which construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene, the method comprising the steps of:

(a) generating a first fragment of the VWF gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain at least one of the mutations;

(b) generating, from the gene or from the first fragment,

a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;

(c) mixing the first and second fragments in the presence
5 of the first, second and third primers;

(d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and

(e) amplifying the extended strand.

10 The extended strand may be amplified to produce a double strand. The method may further comprise the step of isolating the extended strand or its double stranded amplification product, preferably by electrophoresis. The third primer may be a sense or anti-sense primer. In a
15 preferred embodiment, whichever of the first or second primer the third primer pairs with, they may be positioned on either side of the part of the nucleotide sequence which may contain at least one of the mutations.

In one embodiment of the invention, the method may
20 further comprise the step of cloning the synthetic construct into a plasmid vector.

Preferably the third primer has the following sequence:
5'-CTCTTAGGTCCAAAGGCATGAGAAC-3' [SEQ ID No:19], known as VWF
Ex 19:3. The first primer may have the sequence 5'-
25 AGGAGGGCTTTAGATCAGTCACTG-3' [SEQ ID No:20], known as VWF Ex
19:1. The second primer may have the sequence
5'-CAAGTGTTCAGCCAATCTTCACTG-3' [SEQ ID No:21], known as VWF
Ex 19:2. However, any suitable primers may be used.

According to a further aspect of the present invention, there is provided a test kit for examining the human VWF gene, the kit comprising:

- 5 (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being
10 such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether at least one of the mutations including at least a C to T transition at position 2696 and/or a T to A transversion at position 2701 in the
15 coding sequence of the cDNA is present; and
- (c) a control DNA and/or control PCR amplification product, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene,
20 and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene.

According to a further aspect of the present invention,
25 there is provided a method of simultaneously examining a plurality of regions of genomic DNA comprising the steps of:
(a) forming a plurality of different fragments of the genomic DNA, which fragments may contain a known mutation or

polymorphism, wherein each different fragment is formed by a different set of primers;

(b) combining the fragments with a plurality of synthetic nucleotide constructs, each synthetic construct being
5 capable of forming a duplex with a fragment of the genomic DNA;

(c) permitting duplex formation; and

(d) separating the different duplexes formed,
and wherein each set of primers function under the same
10 conditions.

Preferably, each set of primers do not cross-react with each other.

The conditions under which the primers function are preferably PCR cycle conditions.

15 The separation of the duplexes is preferably by electrophoresis, for example gel electrophoresis, and the synthetic constructs are such that the duplexes are well resolved in a single lane on an electrophoretic gel.

In one embodiment, the method may be used to examine
20 simultaneously the factor V gene, MTHFR gene and the gene encoding prothrombin, in particular the mutations referred to in the individual tests mentioned above. In such a case, the primers may be 23 to 25 nucleotides in length. The 3' ends of the primers may be located such that there is no
25 self-hybridisation or cross-hybridisation between them. Typical PCR cycle conditions may be: about 95°C for about 30 seconds; about 63°C for about 30 seconds; about 72°C for about 1 minute for about 30 cycles, terminating with about

95°C for about 5 minutes and about 63°C for about 2 minutes.

The synthetic constructs may take the form as mentioned above in each of the individual examinations of the respective genes and may be in the form of the clones.

5 Alternatively, the method may be used to examine simultaneously the human F8 gene polymorphisms mentioned above, that is preferably the BclI RFLP in intron 18 and the intron 7 polymorphism and in particular the specific polymorphisms mentioned above. The synthetic constructs in
10 such an instance may take the form of those mentioned in the individual F8 examinations above and may be in the form of the clones.

 The method may alternatively be used to examine simultaneously the mutations in the VWF gene mentioned
15 above. In such an instance, the synthetic constructs may take the form of each of those used in the individual VWF examinations above and may be in the form of the clones.

 Preferably, the primer sequences are the same as the first and second primers as described above.

20 Whilst the invention has been described above, it extends to any inventive combination of the features set out above or in the following description.

 The invention will now be described, by way of examples, with reference to the following figures, and in
25 which:

 Figure 1 is a diagram showing the principle of heteroduplex generator construction using the PCR;

 Figure 2 shows heteroduplex profiles obtained for the

factor V G1691A mutation, the prothrombin G20210A variant and the MTHFR C677T mutation using genomic DNAs;

Figure 3 shows a multiplex heteroduplex analysis of genomic DNAs possessing different combinations of FV, PT and MTHFR genotypes;

Figures 4a and 4b respectively show individual and multiple genotyping of the BclI RFLP and intron 7 polymorphism using heteroduplex analysis;

Figures 5a and 5b show the detection of VWD type 2N mutations in exon 20 of the VWF gene by heteroduplex analysis using pVWFex20-UHG1 and pVWFex20-UHG2;

Figure 6 shows the detection of VWD type 2N mutations in exon 19 of the VWF gene by heteroduplex analysis using pEx19-UHG1; and

Figures 7A and 7B show a multiplex heteroduplex analysis of exon 19 and exon 20. Figure 7A shows multiplex analysis using pEx19-UHG1 + pEx20-UHG1 and Figure 7B shows multiplex analysis using pEx19-UHG1 and pEx20-UHG2.

Example 1: Heteroduplex Generator Construction

In the approach to HG synthesis shown in Figure 1, only 3 oligonucleotides of approximately 24 bases in length are required for the synthesis of HG's ranging in size from tens of bases (eg 80bp) to several kilobases (eg 8kb). The principle of this approach is as follows.

Referring to Figure 1, two primers are designed to amplify the target region of genomic DNA and also the HG

(primers 1 and 2). A third primer is designed which may contain the HG identifier and either the normal or the mutant nucleotide (primer 3). It should be noted, however, that the third primer does not have to contain the mutation site. There then follows 3 rounds of PCR synthesis: In the first round, primers 1 and 2 are used to generate a PCR product off genomic DNA (product A); in the second round, product A is used as the substrate for a PCR reaction containing primers 3 and 2. This generates a smaller product (product B) which contains the identifier specified by primer 3; in the third round, product A and product B are mixed and used as the substrates in a PCR containing all three primers, 1, 2 and 3. In this mixed PCR, all of the expected syntheses occur: primers 1 and 2 amplify product A; primers 3 and 2 amplify product B; primers 3 and 2 hybridise to product A and generate more of product B. However, an additional, novel synthesis occurs and it is this which gives rise to the HG. One strand of product B hybridises to the complementary strand of product A and the polymerase extends the B strand to the end of the A strand. The newly synthesised strand is complementary to the A strand except that it contains the identifier originally specified by primer 3. This new strand gets amplified by primers 1 and 2 in subsequent rounds of PCR and a double-stranded PCR product is produced: this is the HG.

It should be noted that primer 3 can be designed to pair either with primer 2, in which case it is a "sense" primer, or to pair with primer 1, in which case it is an

"anti-sense" primer. The principle of the HG synthesis is identical, irrespective of which of these choices is made. Similarly, in the second round of PCR, product B can be synthesised using genomic DNA as substrate for primers 2 and 3 instead of product A.

Electrophoresis of the PCR products in a suitable gel system resolves product A, product B and the HG such that the HG can be quantitatively recovered, for example by excision of the relevant area of gel and diffusion out of the gel into water. The isolated HG can then be cloned into a suitable plasmid vector in a suitable host bacterium. Recombinant bacteria from the cloning experiment are screened for the presence of plasmid containing the HG. The plasmid DNA from a number of appropriate clones is isolated and sequenced to confirm the identity and sequence of the HG. One or more clones are then chosen, glycerol stocks are made and the clone is stored at -70° for subsequent use. The clone represents a theoretically unlimited supply of the HG construct.

The recombinant plasmid containing the HG is used directly in the diagnostic test, there is no necessity to release the HG from the plasmid. It is sufficient to carry out a plasmid mini-prep from an overnight culture of the clone and use the plasmid so obtained directly in the diagnostic test. However, a protocol permitting a larger scale preparation of plasmid of purer quality can also be used.

The procedure detailed above was used to produce the

following HG's and their respective clones:

<u>HG</u>	<u>Clone</u>
FVL Megadel	pFVL Megadel
5 MTHFR-HG1	pMTHFR-HG1
F8 I18-HG1	pF8 I18-HG1
F8 I7-HG1	pF8 I7-HG1
F8 I7-HG2	pF8 I7-HG2
PT HG-3'	pPT-HG1
10 VWFex19-UHG1	pVWFex19-UHG1
VWFex20-UHG1	pVWFex20-UHG1
VWFex20-UHG2	pVWFex20-UHG2

The sequences of primers 1, 2 and 3 used for the generation of each of the above are as follows:

15	<u>HG</u>	<u>Primers</u>
	FVL Megadel	1: 5'-CATGAGAGACATCGCCTCTG-3' [SEQ ID No:2]
		2: 5'-GACCTAACATGTTCTAGCCAGAAG-3'
		[SEQ ID No:3]
		3: 5'-AAGAGCAGATCCCTGGCGAGGAATACAGGTACTT-3'
		[SEQ ID No:1]
20		
	MTHFR-HG1	1: 5'-AGGGAGCTTTGAGGCTGACCTGAA-3'

[SEQ ID No:5]

2: 5'-ACGATGGGGCAAGTGATGCCCATG-3'

[SEQ ID No:6]

3: 5'-GAAGGAGAAGGTGTCTGCGGCCGATTTTCATCATCA-3'

5

[SEQ ID No:4]

F8 I18-HG1

1: 5'-TAAAAGCTTTAAATGGTCTAGGC-3'

[SEQ ID No:11]

2: 5'-TTCGAATTCTGAAATTATCTTGTTTC-3'

[SEQ ID No:12]

10

3: 5'-CAGTGATCAGATCAAGCTCCATGCTC-3'

[SEQ ID No:10]

F8 I7-HG1

1: 5'-AACAGCCTAATATAGCAAGACACTC-3'

[SEQ ID No:14]

2: 5'-CACATCCATTTCAGAATCAGTAAG-3'

15

[SEQ ID No:15]

3: 5'-AGCAAGACACTCCCCTGACATTGCTTTGG-3'

[SEQ ID No:13]

PT HG-3'

1: 5'-GTGTTTCTAAAACTATGGTTCCCAA-3'

[SEQ ID No:8]

20

2: 5'-CCCAGAGAGCTGCCCATGAATAG-3'

[SEQ ID No:9]

3: 5'-GACTCTCAGCGCTCAATGCTC-3'

[SEQ ID No:7]

VWFex19-UHG1

1: 5'-AGGAGGGCTTTAGATCAGTCACTG-3'

[SEQ ID No:20]

2: 5'-CAAGTGTTCAGCCAATCTTCACTG-3'

[SEQ ID No:21]

3: 5'-CTCTTAGGTCCAAAGGCATGAGAAC-3'

5

[SEQ ID No:19]

VWFex20-UHG1 1: 5'-CAACTTGTCATCTCTGCCATGACTG-3

[SEQ ID No:17]

2: 5'-CTCACCTGCACCAGAACGTACTGG-3'

[SEQ ID No:18]

10

3: 5'-GTCGGGACCGGTGGAAGTGCAC-3'

[SEQ ID No:16]

In the case of pFVL Megadel, pF8 I7HG-1 and pVWFex20-UHG2, the process of PCR followed by cloning introduced a sequence change which was not specified by primer 3. These clones were, however, chosen instead of clones containing the authentic, pre-specified sequences since the profiles they produce in the relevant diagnostic tests are much better than the profiles obtained from the purposefully designed sequences. Put succinctly, artifactual sequence changes resulted in variant HG's which improved upon the original design. These are discussed in the relevant sections below.

The following clones were also generated containing control PCR products relevant to each HG:

25 HGClone

	FVL Megadel	pFVL (G)
		pFVL (A)
	MTHFR-HG1	pMTHFR (C)
		pMTHFR (T)
5	PT HG-3'	pPT (G)
		pPT (A)
	F8 I18-HG1	pF8 I18 (T)
		pF8 I18 (A)
	F8 I7-HG1	pF8 I7 (G)
10		pF8 I7 (A)
	VWFex19-UHG1	pVWFex19 (R53)
		pVWFex19 (W53)
	VWFex20-UHG1	pVWFex20 (R91)
		pVWFex20 (Q91)
15		pVWFex20 (R89)
		pVWFex20 (Q89)

The control clones are listed again for clarity at the end of each relevant section.

Example 2

Background To Diagnostic Tests

Example 2A: Factor V Leiden

Coagulation factor V (FV) is one of several essential proteins of the coagulation cascade. In its activated form (FVa) it serves as cofactor to activated factor X (FXa) in the activation of prothrombin to thrombin. FVa is inactivated by activated protein C (APC) which cleaves FVa at 3 sites (Arg306, Arg506 and Arg679). Cleavage at Arg506 is a pre-requisite for subsequent cleavage at the other two sites, which is otherwise inefficient. Relatively recently, a variant of FVa has been found which is resistant to cleavage by APC at Arg506. This APC-resistant variant can not be efficiently inactivated and this leads to a prothrombotic state. The variant arises as a result of a single nucleotide substitution (point mutation) in the FV gene. The point mutation occurs in codon 506 and is a G to A transition which converts the triplet CGA, encoding arginine, to CAA, encoding glutamine. Whereas APC can cleave FVa at position 506 when the amino acid present is arginine, it can not cleave when the amino acid present is glutamine. This explains the APC-resistance of the variant FVa. The implicated nucleotide occurs at position 1691 in the FV mRNA and, using standard nomenclature, the mutation is represented G1691A (G = normal, A = variant). It has become known as the FV Leiden mutation after the city in

which the research which led to its characterisation was conducted.

Several genetic tests for FV Leiden have been described. All use the PCR followed by various approaches
5 to product analysis. For example, one approach takes advantage of the fact that FV Leiden abolishes an MnlI restriction site in the DNA: the PCR product is digested with MnlI and the digestion profile indicates the genotype of the test DNA. This approach suffers the disadvantage
10 that mutation of any of the four nucleotides in the MnlI recognition sequence will give the same result as FV Leiden. It is therefore not 100% specific for the FV Leiden mutation. Along with a number of the other approaches, it also suffers the disadvantage that there is significant post-PCR
15 sample manipulation. Lack of specificity and laborious sample handling are adverse features for a routine diagnostic test requiring high sample throughput.

Heteroduplex analysis provides a suitable alternative approach. The HG is simply mixed with the patient's DNA (at
20 the correct relative quantity) and then a PCR is carried out to co-amplify the genomic and HG targets. Heteroduplexes form between genomic PCR strands and HG PCR strands as a natural result of the cyclical denaturation and hybridisation of the PCR. Therefore, at the end of PCR, an
25 aliquot of the products is electrophoresed directly on a suitable gel system without further manipulation. The conformation of the heteroduplexes depends upon whether the normal G nucleotide or FV Leiden A nucleotide is present and

the conformational differences result in electrophoretic mobility differences. The electrophoresis profile therefore defines the genotype of the sample. Since the FV gene is autosomal (chromosome 1), there are 2 copies per somatic
5 cell and the genotype may be G+G, G+A, or A+A. Each genotype gives a distinct and diagnostic profile in the heteroduplex region of the gel.

We tried a variety of HG identifier designs eg. deletions of 3 to 5 nucleotides positioned between 1 and 3
10 nucleotides away from the FV Leiden site. An identifier comprising a 3 nucleotide deletion spanning nucleotides -4 to -2 on the 5' side of the mutation site (nucleotide 0) has been used. However, following subsequent research, an improved diagnostic profile was obtained with a different
15 HG. The identifier in this HG was a 5 nucleotide deletion corresponding to nucleotides -6 to -2 relative to the FV Leiden site (Fig 2).

When this HG was cloned, several clones were screened and the recombinant plasmid in one clone gave a profile
20 different to and better than the original profile (Fig 2). When the HG in this clone was sequenced, it contained the pre-specified 5 nucleotide deletion, but additionally it contained an inserted C nucleotide 7 bases 5' to the FV Leiden site. The additional C nucleotide was a fortuitous
25 PCR-cloning artifact which gave rise to an improved diagnostic heteroduplex profile for FV Leiden. This HG was called "FVL Megadel" and the clone "pFVL Megadel".

Figure 2 shows heteroduplex profiles obtained for the

factor V G1691A mutation (FV), the prothrombin G20210A variant (PT) and the methylene tetrahydrofolate reductase (MTHFR) C677T mutation using genomic DNAs of known genotype indicated by G/G, G/A, A/A, C/C, C/T or T/T as appropriate.

5 The heteroduplex profiles obtained with two FV HGs are shown for comparison: pFVdel5, in which the HG contained a specified deletion of 5 nucleotides, and pFVL-Megadel, in which an artifactual insertion of a C nucleotide occurred 7 bases upstream of the specified 5 nucleotide deletion. The

10 heteroduplex profiles for the prothrombin variant and the MTHFR mutation were obtained using pPT-HG1 and pMTHFR-HG1 respectively. Arrows (►) indicate the positions of homoduplex PCR products (approximately 147 bp for the FV and MTHFR PCRs, approximately 90 bp for the PT PCR). Lane m = DNA

15 size standard pBR322/MspI.

Below are indicated the sequence of the genomic PCR product from which the HG was made and the sequence of the HG (primer sequences are as indicated above):

Factor V: Normal Genomic PCR Sequence [SEQ ID No:22]

20 CATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGATC

CCTGGACAGGCGAGGAATACAGGTACTTTGTCCTTGAAGTAACCTTTCAGAAATTCT

GAGAATTTCTTCTGGCTAGAACATGTTAGGTC

Heteroduplex Generator: Megadel Sequence [SEQ ID No:23]

CATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGATC

CCCTGGCGAGGAATACAGGTACTTTGTCCTTGAAGTAACCTTTCAGAAATTCT

5 GAGAATTTCTTCTGGCTAGAACATGTTAGGTC

Primer sequences can be seen in [SEQ ID No: 22] and [SEQ ID No. 23]. ACAGG in [SEQ ID No: 22] indicate the 5 nucleotides deleted in [SEQ ID No: 23] by primer 3. Bold 'C' in [SEQ ID No: 23] indicates the artefactual 'C' insertion in
 10 Megadel (see text). FVL-del5 and its cloned form pFVLdel5 lack this 'C' insert.

Example 2B: Methylenetetrahydrofolate Reductase.

Homocysteine is derived metabolically only from the essential amino acid methionine. Hyperhomocysteinemia is an
 15 independent risk factor for peripheral and coronary arterial disease, stroke and venous thrombosis. Methylenetetrahydrofolate reductase (MTHFR) is one of the major enzymes involved in the development of hyperhomocysteinemia. One variant of this enzyme, thermolabile MTHFR, is due to a C to

T transition at nucleotide 677 of the coding sequence, a mutation which, in homozygous form, can lead to significantly elevated plasma homocysteine levels. The frequency for homozygosity varies greatly from 5-12% among Caucasian populations. The mutation creates a HinfI and TaqI restriction site in the DNA sequence and can be detected by PCR followed by HinfI or TaqI digestion.

We have investigated the use of heteroduplex analysis for detection of C677T in the MTHFR gene. A heteroduplex generator was designed of length 144bp in which the identifier was a 3 nucleotide deletion comprising nucleotides -4 to -2 relative to the MTHFR mutation site (nucleotide 0). The genomic PCR was designed to give a product of 147bp and the 5' and 3' extremes were positioned such that the mutation site resided 54 nucleotides downstream of the 5' end. This HG gave a very clear distinction between normal and mutant nucleotides on heteroduplex analysis and the three genotypes C+C, C+T and T+T were easily distinguishable (Fig 2). The HG ("MTHFR-HG1") was cloned and recombinant plasmid was isolated from an appropriate clone and sequenced to confirm its identity. The selected clone was given the identity "pMTHFR-HG1".

Below are indicated the sequence of the genomic PCR product from which the HG was made and the sequence of the HG (primer sequences are as indicated above):

Methylene Tetrahydrofolate Reductase: Genomic PCR Sequence
[SEQ ID No:24]

AGGGAGCTTTGAGGCTGACCTGAAGCACTTGAAGGAGAAGGTGTCTGCGGGAGCCG

ATTCATCATCACGCAGCTTTTCTTTGAGGCTGACACATTCTTCCGCTTTGTGAAGGC

ATGCACCGACATGGGCATCACTTGCCCCATCGT

Heteroduplex Generator: MTHFR-HG1 Sequence [SEQ ID No:25]

5 AGGGAGCTTTGAGGCTGACCTGAAGCACTTGAAGGAGAAGGTGTCTGCGGCCG

ATTCATCATCACGCAGCTTTTCTTTGAGGCTGACACATTCTTCCGCTTTGTGAAGGC

ATGCACCGACATGGGCATCACTTGCCCCATCGT

Primer sequences can be seen in [SEQ ID No: 24] and [SEQ ID No: 25]. GGA in [SEQ ID No: 24] indicate the 3 nucleotides
10 deleted in [SEQ ID No:25] by primer 3.

Example 2C: Prothrombin.

Prothrombin (PT) is the inactive precursor of thrombin, a coagulation factor with multiple actions in haemostasis.

These range from the conversion of fibrinogen to fibrin, positive feedback activation of factors V and VIII, activation of factor XIII and platelets, to negative feedback regulation via the protein C pathway. The gene encoding prothrombin has been localised to chromosome 11 and a nucleotide substitution in the 3' transcribed but untranslated region has been found to be associated with elevated levels of circulating prothrombin. The substitution is the transition of G at position 20210 in the gene to A and occurs at low frequency in the general population (1%) but at elevated frequency among patients with venous thrombosis (18%). It has not yet been established whether the G20210A transition is causative of increased PT levels, or whether it resides on a PT allele in which another sequence change results in elevated circulating PT (allelic association). However, its presence signifies an increased genetic risk for venous thrombosis based upon its elevated frequency in thrombosis cohorts. The nucleotide substitution is currently detected by PCR followed by HindIII restriction endonuclease digestion. The G20210A transition does not alter a restriction site in the DNA, therefore a mutagenic PCR primer is used which creates a HindIII recognition sequence if the A nucleotide, which is associated with thrombosis, is present.

We investigated the use of heteroduplex analysis for the detection of this mutation. An initial HG was designed of length 87bp, with a 3 nucleotide deletion from positions +2 to +4 on the 3' side of the mutation site (nucleotide 0).

The mutation site was positioned 43 nucleotides downstream of the 5' end of the 87bp genomic PCR product. This HG did not give an acceptable diagnostic profile and a systematic process of redesign followed by experimentation culminated in a HG of length 82 bp with a 3 nucleotide deletion from positions +1 to +3 relative to the mutation site. The genomic PCR also had to be redesigned to give a product of length 85bp in which the mutation site was located 43 nucleotides downstream of the 5' end.

10 The final HG gave good differentiation between G and A forms in genomic DNA and permitted all three genotypes G+G, G+A, A+A to be clearly distinguished (Fig 2). The HG was cloned and sequenced to confirm its identity (clone pPT-HG1).

15 Below are indicated the sequence of the genomic PCR product from which the HG was made and the sequence of the HG (primer sequences are as indicated above):

Prothrombin: Genomic PCR Sequence [SEQ ID No:26]

GTGTTTCTAAAACTATGGTTCCCAATAAAAGTGACTCTCAGCGAGCCTCAATGCTCC

20 CAGTGCTATTCATGGGCAGCTCTCTGGG

Heteroduplex Generator: PT-HG1 Sequence [SEQ ID No:27]

GTGTTTCTAAAACTATGGTTCCCAATAAAAGTGACTCTCAGCGCTCAATGCTCC

CAGTGCTATTCATGGGCAGCTCTCTGGG

Primer sequences can be seen in [SEQ ID No: 26] and [SEQ ID
5 No: 27]. AGC in [SEQ ID No: 26] represent the 3 nucleotides
deleted in [SEQ ID No: 27] by primer 3.

Example 2D: A Single Test For Factor V Leiden G1691A,
Methylenetetrahydrofolate Reductase C677T and Prothrombin
G20210A Combined.

10 The HG's described above for each of the three diagnostic
tests FVLeiden, MTHFR and PT had an additional component in
their design: they were purposefully designed to function
together in a single, multiplex test, without cross inter-
ference. To achieve this, the following criteria had to be
15 incorporated in the design process:

- i. The three pairs of primers required for the 3 tests collectively should function in the same PCR cycle conditions.
- ii. The three pairs of primers should not cross-react with
20 each other in any way.

iii. The three HG's should give heteroduplex profiles which are well resolved in a single lane on an electrophoretic gel so that there is no ambiguity in the interpretation of the result for each test.

5 The accomplishment of the above was a major challenge which was achieved as follows:

i. The primers were designed to be 23-25 nucleotides in length to permit stringent conditions in the PCR. This, in turn, permitted specificity of each PCR on genomic DNA and
10 on the HG's.

ii. The 3' ends of the primers were located such that there would be no self hybridisation or cross hybridisation yielding non-specific products, such as primer dimers.

iii. The identifiers of the three HG's were purposefully
15 chosen: For FV Leiden the identifier was a 5 nucleotide deletion, whilst for MTHFR and PT it was a 3 nucleotide deletion. The principle here was that other research within our laboratory had shown that deletion size influences the position of the heteroduplexes on a gel in a predictable
20 manner, therefore this parameter was used to position the heteroduplexes for each test at an approximate position on the gel. For MTHFR and PT, although the identifier was identical (a deletion of 3 nucleotides), the size of the PCR products differed (respectively 147bp and 85bp), thereby

placing the results for each test at different positions on the gel (Figure 3).

The specific design components which permitted the three tests to be combined were therefore: the sequence of
 5 the primers used for each of the three PCR's, the size of each PCR product and the nature of the HG identifier.

Several clones were generated to provide controls for these three genetic tests:

	<u>Control for:</u>	<u>Clone</u>
10	Normal FV	pFVL(G) .
	FV Leiden	pFVL(A) .
	Normal MTHFR	pMTHFR(C) .
	Mutant MTHFR	pMTHFR(T) .
	Normal prothrombin	pPT(G) .
15	Variant prothrombin	pPT(A) .

Figure 3 shows multiplex heteroduplex analysis of 7 genomic DNAs (lanes a-g) possessing different combinations of factor V (FV), prothrombin (PT) and methylene tetrahydrofolate reductase (MTHFR) genotypes as follows:

20	Lane	FV	MTHFR	PT
	a	G G	C C	G G
	b	A A	C C	G G
	c	G G	C C	A A

d	G G	T T	G G
e	G A	C T	G A
f	A A	T T	G G
g	G A	T T	G G

- 5 Arrows (►) indicate the positions of homoduplex PCR products (approximately 147 bp for the FV and MTHFR PCRs, approximately 90 bp for the PT PCR). Lane m = DNA size standard pBR322/MspI.

Factor 8 Genetic Markers.

- 10 Example 2E: The BclI Restriction Fragment Length Polymorphism.

- Coagulation factor 8 is a key protein cofactor of the coagulation cascade and a deficiency or dysfunction of the molecule gives rise to Haemophilia A. In the majority of cases, the deficiency or dysfunction is due to a defect in the gene encoding factor 8. This gene is carried on the X chromosome and Haemophilia A is an X-linked, recessive disorder which is carried by females and which presents in males. It affects approximately 1 in 10,000 males in the general population.

Families in which an affected individual occurs may require genetic investigation with a view to establishing carrier status in "at risk" females or prenatal diagnosis for established carriers. Currently, such genetic investi-

gations involve linkage analysis - the use of genetic markers located within the factor 8 gene to track a defective or normal gene through a family.

One of the most commonly used genetic markers is the BclI restriction fragment length polymorphism (RFLP) in intron 18 of the factor 8 gene. This is a dimorphic genetic marker which is due to a single nucleotide difference between factor 8 genes: the majority (75%) contain a T nucleotide at the relevant position in intron 18, the rest (25%) contain an A nucleotide at that position. The dimorphic nucleotide occurs within the sequence TGATCA, which is the recognition sequence for the restriction endonuclease BclI. When T is present, BclI will cut the DNA, when A is present (TGAACA), BclI will not cut the DNA.

The current approach for analysis of this genetic marker is to amplify a 142bp stretch of genomic DNA spanning the BclI site and then digest the PCR product with BclI. If T is present, digestion products of 99bp+43bp are obtained, however, if A is present, the PCR product is not digested and remains at 142bp.

We have investigated the use of heteroduplex analysis to analyse the BclI locus. A HG was designed which contained a 5 nucleotide deletion representing nucleotides +2 to +6 on the 3' side of the dimorphic site (nucleotide 0). The identifier also contained a T insertion between nucleotides +9 and +10 relative to the mutation site in genomic DNA; in the HG, the corresponding insertion point is +5 and +4. The T insertion was based on other research carried

out in our laboratory which suggested that the conformations which heteroduplexes could adopt might be minimised by the inclusion of a second, small change in the vicinity of the primary identifier. This change might "force" the most stable conformation to be obtained when several stable conformations are possible. This HG gave clear distinction between the T and A forms of the BclI RFLP and permitted unequivocal determination of all possible genotypes (males: T or A; females: T+T, T+A, or A+A) (Figure 4A). This HG ("F8 I18HG-1") was cloned and sequenced to confirm its identity (clone pF8 I18HG-1).

Below are indicated the sequence of the genomic PCR product from which the HG was made and the sequence of the HG (primer sequences are as indicated above):

15 F8 BclI RFLP: Genomic PCR Sequence [SEQ ID No:28]

TAAAAGCTTTAAATGGTCTAGGCACTGGGAACACAATCAGTGATCAAAGCAGA

CAAGCTCCATGCTCTTAAGTAATACTTTTTCAAATGCTTTTTAAACCATTGTTC

AAAGCAAATGAACAAGATAATTCAGAATTCGAA

Heteroduplex Generator: F8 I18-HG1 Sequence [SEQ ID No:29]

TAAAAGCTTTAAATGGTCTAGGCACTGGGAACACAATCAGTGATCAGAT

CAAGCTCCATGCTCTTAAGTAATACTTTTTCAAATGCTTTTTTAAACCATTGTTC

5 AAAGCAAATGAACAAGATAATTTTCAGAATTCGAA

Primer sequences can be seen in [SEQ ID No:28] and [SEQ ID No: 29]. AAAGC in [SEQ ID No: 28] indicate the 5 nucleotides deleted in [SEQ ID No: 29] by primer 3. Bold 'T' in [SEQ ID No: 29] indicates the 'T' insert also specified by primer 3.

10

Example 2F: The Factor 8 Intron 7 Polymorphism.

Intron 7 of the human factor 8 gene contains a dimorphic nucleotide position. The majority (80%) of factor 8 genes possess a G nucleotide at this position, the remainder possess an A (20%). A restriction endonuclease sequence is not affected by either nucleotide.

15

The current approach for its analysis is to use a PCR in which the sequence of the upstream primer is altered such that an AlwNI recognition sequence is created if G is

present in the genomic template but not if A is present. After PCR, the PCR product (175bp) is digested with AlwNI and fragments of 150bp+25bp are obtained if G is present, whilst the product remains undigested at 175bp if A is present.

We applied heteroduplex analysis to the investigation of this dimorphic site and designed a HG of 177bp in which the identifier was an insertion of 3 'C' nucleotides between positions -1 and -2 on the 5' side of the dimorphism (nucleotide 0). Additionally, an insertion of a single C nucleotide was made between positions +6 and +7 on the 3' side of the dimorphism for the reasons explained above in the section describing heteroduplex analysis of the BclI RFLP.

The HG was cloned and one clone gave a heteroduplex profile which was different from, and better than, that obtained from the other clones tested (Figure 4A). This clone contained an artefactual C to T substitution at the middle nucleotide of the CCC insert. The clone containing the designed HG was called pF8I7 HG-2; the clone containing the C to T substitution was called pF8I7HG-1.

The clone pF8 I7HG-1 was selected for subsequent use because it gave clearer differentiation between the genotypes: G or A (males) and G+G, G+A, or A+A (females) compared with pF8I7HG-2.

Figure 4 shows genotyping the BclI RFLP and intron 7 polymorphism using heteroduplex analysis.

Figure 4A shows analysis of the two polymorphic loci

individually. DNA samples of known genotype were used: hemizygous BclI (+), heterozygous BclI (+/-), hemizygous BclI (-) and hemizygous intron 7 (G), heterozygous intron 7 (G/A), hemizygous intron 7 (A). The intron 7 G/A heteroduplex profiles obtained with two HGs are compared: p17-HG1, which contains the designed HG and which was common to several clones, and p17-HG2, which contains a mutated HG. The BclI lanes contain a non-specific smear (▲) close to the slower migrating heteroduplex band of the (-) allele (●), however this does not interfere with the interpretation of the profile. Lane m = pBR322/MspI DNA size standards.

Below are indicated the sequence of the genomic PCR product from which the HG was made and the sequence of the HG (primer sequences are as indicated above):

15 F8 Intron 7 G/A: Genomic PCR Sequence [SEQ ID No:30]

AACAGCCTAATATAGCAAGACACTC~TGACATTG~TTTGGTTTGTCTGACTCCA

GGATGGCATGGAAGCTTATGTCAAAGTAGACAGCTGTCCAGAGGAACCCCAA

CTACGAATGAAAAATAATGAAGAAGCGGAAGACTATGATGATGATCTTACTGA

TTCTGAAATGGATGTG

Heteroduplex Generator: F8 I7-HG1 Sequence [Seq ID No:31]

AACAGCCTAATATAGCAAGACACTCCTCTGACATTGCTTTGGTTTGTCTGACTCCA

GGATGGCATGGAAGCTTATGTCAAAGTAGACAGCTGTCCAGAGGAACCCCAA

CTACGAATGAAAAATAATGAAGAAGCGGAAGACTATGATGATGATCTTACTGA

5 TTCTGAAATGGATGTG

Heteroduplex Generator: F8-HG2 Sequence [SEQ ID No:32]

AACAGCCTAATATAGCAAGACACTCCCCTGACATTGCTTTGGTTTGTCTGACTCCA

GGATGGCATGGAAGCTTATGTCAAAGTAGACAGCTGTCCAGAGGAACCCCAA

CTACGAATGAAAAATAATGAAGAAGCGGAAGACTATGATGATGATCTTACTGA

5 TTCTGAAATGGATGTG

Primer sequences can be seen in the sequences (primer 3 overlaps primer 1). The 3 'C' nucleotides in bold in [SEQ ID No: 32] have been inserted by primer 3 into the sequence in [SEQ ID No: 30] at -, giving pI7-HG2. The middle 'C' of
 10 this trinucleotide insert was mutated to a 'T' in pI7-HG1 ([SEQ ID No: 31] above and see text).

Example 2G: Combined Genetic Testing Of The Factor 8 BclI RFLP In Intron 18 And The Intron 7 Polymorphism.

The heteroduplexes produced for genotyping the BclI RFLP and
 15 the intron 7 polymorphism were designed to permit both tests to be done in a single PCR and to permit the products of each test to resolve from one another in a single lane on

gel electrophoresis (Figure 4B). The cloning artifact which gave rise to the final intron 7 construct did not interfere with this plan. Indeed, it enhanced the intron 7 diagnostic profile without altering the position of the intron 7 result
 5 on the gel.

HG design and synthesis for both the BclI RFLP and the intron 7 polymorphism would not have been possible without establishing the sequence of the respective 142bp and 175bp genomic products since these sequences have not been
 10 published by other researchers.

Several clones were generated to provide controls for the above genotyping tests:

<u>Control for:</u>	<u>Clone</u>
<u>BclI</u> T	pF8 I18(T).
15 <u>BclI</u> A	pF8 I18(A).
Intron 7 G	pF8 I7(G).
Intron 7 A	pF8 I7(A).

Figure 4B shows multiplex analysis of the intron 18 BclI and intron 7 G/A polymorphisms. DNA samples with the
 20 following genotypes were used: Lanes a and d, BclI (+), intron 7 (G); Lanes b and e, BclI (+/-), intron 7 (G/A); Lanes c and f, BclI (-), intron 7 (A).

The multiplex profiles obtained with p17-HG1 and p17-

HG2 are shown for comparison. A non-specific smear (▲) associated with the BclI test does not interfere with the interpretation of the profile. Lane m = pBR322/MspI DNA size standard.

5 Example 2H: Von Willebrand Disease Type 2N.

Von Willebrand factor (VWF) is a plasma glycoprotein with several biological functions relevant to haemostasis. Included among these is its role as the carrier molecule for coagulation factor 8 (F8). Mutations which bring about
 10 amino acid substitutions in the F8 binding domain of VWF can interfere with F8 binding and can result in decreased levels of circulating F8. Inefficient haemostasis due to decreased F8 levels resulting from mutations in the region of the F8 gene encoding the F8 binding domain is known as von Willebr-
 15 and disease type 2N. Several mutations have been described in type 2N VWD, however two mutations predominate among affected patients. These are G2811A (R91Q), which is located in exon 20 of the VWF gene, and C2696T (R53W)²⁰, which is located in exon 19 of VWF gene. Between 80 to 85%
 20 of patients possess one or both of these two mutations.

Routine screening for these mutations is not yet undertaken in any laboratory, however there are a number of centres nationally and internationally in which such screens are in the developmental phase. The standard approach is
 25 PCR followed by nucleotide sequence analysis in order to obtain definitive mutation characterisation.

We have applied heteroduplex analysis to the detection of G2811A (R91Q) and C2696T (R53W). Our approach was not limited to the detection of these two mutations only. The common G2811A (R91Q) mutation is close to a second type 2N mutation G2823T (C95F). Similarly, the common mutation C2696T (R53W) is close to another type 2N mutation T2701A (H54Q). Our strategy was to design two HG's, one of which would detect G2811A (R91Q) and G2823T (C95F), whilst the other would detect C2696T (R53W) and T2701A (H54Q).

For G2811A (R91Q) and G2823T (C95F) the identifier of the HG was placed between the two mutation sites. The identifier was a 3 nucleotide deletion eliminating nucleotides +2 to +4 on the 3' side of G2811A. These 3 nucleotides correspond to nucleotides -8 to -10 on the 5' side of G2823T (C95F). This HG ("VWFex20-UHG1", length 173bp) was synthesised from a genomic PCR product of length 176bp by deletion of nucleotides 49 to 51 of the genomic PCR product.

VWFex20-UHG1 gave clear distinction between G2811A (R91Q) and the normal sequence and between G2823T (C95F) and the normal sequence (Figure 5A). Additionally, the heteroduplex profiles for G2811A and G2823T were distinct from one another, thereby permitting genetic diagnosis of either mutation using the single HG. VWFex20-UHG1 also detected a previously reported polymorphism of the VWF gene, G2805A (R89Q). The heteroduplex profiles for the polymorphism were different to those of the mutations G2811A and G2823T, therefore it is possible to determine the genotype of all three loci with this single HG.

For the mutations C2696T (R53W) and T2701A (H54Q) the HG identifier was placed between the two mutation sites and consisted of the insertion of 3 A nucleotides between position 0 and +1 relative to C2696T (position 0), corresponding to positions -5 and -4 relative to T2701A. The insertion was made using a genomic PCR product of length 162bp and was located between nucleotides 62 and 63 of the genomic product.

The resulting 165bp HG ("VWFex19-UHG1") gave clear distinction between the two mutations C2696T (R53W) and the normal sequence and also between T2701A (H54Q) and the normal sequence. Additionally, the heteroduplex profiles of C2696T and T2701A differed, thereby permitting genetic diagnosis of either mutation using this single HG (Figure 6).

The two HG's VWFex19-UHG1 and VWFex20-UHG1 were cloned and an appropriate clone of each was sequenced to confirm identity ("pVWFex19-UHG1" and "pVWFex20-UHG1" respectively). The cloning experiment generated a mutated HG which, like VWFex20-UHG1, gave clear distinction between G2811A (R91Q) and the normal sequence and between G2823T (C95F) and the normal sequence (Figure 5B). Additionally, the heteroduplex profiles for each mutation differed, thereby permitting genetic diagnosis of either mutation using this HG. The profiles obtained with this HG ("VWFex20-UHG2") were better positioned on the gel to permit combined investigation of the exon 20 and exon 19 mutation sites (see section "Combined Genetic Diagnosis Of VWD Type 2N Mutations C2696T

(R53W), T2701A (H54Q), G2811A (R91Q) and G2823T (C95F)" below). The clone of VWFex20-UHG2 was stored for future use ("pVWFex20-UHG2").

Figure 5 shows detection of VWD type 2N mutations in exon 20 of the VWF gene by induced heteroduplex formation using: A. pEx20-uHG1, which contains the designed HG; and B. pEx20-uHG2, which contains a mutated HG. DNA samples with the following exon 20 genotypes were used:

	Lane	Type 2N mutation		G2805A	Polymorphism
10		allele 1	allele 2	allele 1	allele 2
	a	none	none	G	G
	b	none	none	G	A
	c	G2811A	G2811A	G	G
	d	G2811A	none	G	G
15	e	G2811A	none	G	A
	f	G2811A	G2823T	G	G
	g	C2810T	none	G	G

Lane m, DNA size standards (pBR322/MspI).

Figure 6 shows detection of VWD type 2N mutations in exon 19 of the VWF gene by induced heteroduplex formation using pVWFEx19-UHG1. Lane a, normal genomic DNA + pVWFEx19-UHG1 PCR products mixed but not denatured and rehybridised. Lanes b-e, DNA samples with the following exon 19 genotypes: b. homozygous normal; c. heterozygous normal + T2701A (H54Q); d. heterozygous normal + C2696T (R53W); e.

homozygous C2696T (R53W). Lane m, DNA size standards
pBR322/MspI.

Below are indicated the sequence of the genomic PCR
product from which the HG was made and the sequence of the
5 HG (primer sequences are as indicated above):

VWF Exon 19 [SEQ ID No:33]

AGGAGGGCTTTAGATCAGTCACTGTGGCCCTGAGGACTTTTGGATTCTTTTCTCTT

AGGTCC~GGCATGAGAACAGATGTGTGGCCCTGGAAAGGTGTCCCTGCTTCCAT

CAGGGCAAGGAGTATGCCCCTGGAGAAACAGTGAAGATTGGCTGCAACACTTG

Heteroduplex Generator: VWFex19-UHG 1 Sequence [SEQ ID No:34]

AGGAGGGCTTTAGATCAGTCACTGTGGCCCTGAGGACTTTTGGATTCTTTTCTCTT

AGGTCCAAAGGCATGAGAACAGATGTGTGGCCCTGGAAAGGTGTCCCTGCTTCCAT

5 CAGGGCAAGGAGTATGCCCCTGGAGAAACAGTGAAGATTGGCTGCAACACTTG

Primer sequences can be seen in [SEQ ID No: 33] and [SEQ ID No: 34]. The 3 'A' nucleotides in bold in [SEQ ID No: 34] have been inserted by primer 3 into the sequence in [SEQ ID No: 33] at-.

10 VWF Exon 20: Genomic PCR Sequence [SEQ ID No:35]

CAACTTGTTCATCTCTGCCATGACTGCTCCTAGTGTCTGTCTGGGACCGGAAGTGGA

ACTGCACAGACCATGTGTGTGATGCCACGTGCTCCACGATCGGCATGGCCCACTA

CCTCACCTTCGACGGGCTCAAATACCTGTTCCCCGGGGAGTGCCAGTACGTTCTGG

TGCAGGTGAG

Heteroduplex Generator: VWFex20-UHG 1 Sequence [SEQ ID
No:36]

CAACTTGTCATCTCTGCCATGACTGCTCCTAGTGTCTGTCGGGACCGGTGGA

ACTGCACAGACCATGTGTGTGATGCCACGTGCTCCACGATCGGCATGGCCCCACTA

5 CCTCACCTTCGACGGGCTCAAATACCTGTTCCCCGGGGAGTGCCAGTACGTTCTGG

TGCAGGTGAG

Heteroduplex Generator: VWFex20-UHG 2 Sequence [SEQ ID
No:37]

CAACTTGTCATCTCTGCCATGACTGCTCCTGGTGTCTGGTCGGGACCGGTGGA

10 ACTGCACAGACCATGTGTGTGATGCCACGTGCTCCACGATCGGCATGGCCCCACTA

CCTCACCTTCGACGGGCTCAAATACCTGTTCCCCGGGGAGTGCCAGTACGTTCTGG

TGCAGGTGAG

Primer sequences can be seen in the sequences. AAG in [SEQ ID No: 35] are the 3 nucleotides deleted in [SEQ ID No: 36] and [SEQ ID No: 37] by primer 3. Bold 'G' nucleotides in [SEQ ID No: 37] represent the artefactual substitutions in VWFex20-UHG2.

Combined Genetic Diagnosis Of VWD Type 2N Mutations C2696T (R53W), T2701A (H54Q), G2811A (R91Q) And G2823T (C95F).

10 The HG's VWFex19-UHG1 and VWFex20-UHG1 and the primers used in their respective PCR's were designed to permit both tests to be done in combination in a single reaction tube. The criteria adopted to achieve this were identical to those given under "A Single Test For factor V Leiden G1691A, 15 Methylenetetrahydrofolate Reductase C677T and Prothrombin G20210A". Multiplex PCR using pVWFex19-UHG1 and pVWFex20-UHG1 and genomic DNA gives the expected heteroduplex profiles for each of the four mutations C2696T (R53W), T2701A (H54Q), G2811A (91Q) and G2823T (C95F) (Figures 7A 20 and 7B). Therefore, all four potential mutation sites can be screened for the presence of a mutation in a single reaction tube. Additionally, the polymorphism G2805A (R89Q) is detected by, and can be genotyped in, this multiplex PCR.

Substitution of pVWFex20-UHG1 by pVWFex20-UHG2 permits identical information to be obtained, however the multiplex profile is better resolved on the gel. Therefore, although either pVWFex20-UHG1 or -UHG2 can be used in the multiplex
 5 diagnosis of certain type 2N VWD mutations, pVWFex20-UHG2 is the construct of choice for this analysis since it provides better resolution of the various profiles on electrophoresis.

If only exon 20 is being investigated, then either
 10 pVWFex20-UHG1 or pVWFex20-UHG2 can be used.

Several clones have been generated to provide positive controls for the type 2N VWD tests:

<u>Control for:</u>	<u>Clone</u>
C at 2696	pVWFex19(R53) .
15 T at 2696	pVWFex19(W53) .
G at 2811	pVWFex20(R91) .
A at 2811	pVWFex20(Q91) .
G at 2805	pVWFex20(R89) .
A at 2805	pVWFex20(Q89) .

20 Figures 7A and 7B show multiplex heteroduplex analysis of exon 19 and exon 20 of the VWF gene using: A. pVWFex19-UHG1 + pVWFex20-UHG1; and B. pVWFex19-UHG1 + pVWFex20-UHG2. The VWF genotypes of the DNA samples used were as follows:

Lane	Exon 19		Exon 20		G2805A Polymorphism	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
a	C2696T	C2696T	normal	normal	A	A
b	T2701A	normal	normal	normal	G	G
c	normal	normal	normal	normal	G	G
d	normal	normal	normal	normal	G	A
e	normal	normal	G2811A	normal	G	G
f	normal	normal	G2811A	G2811A	G	G
g	C2696T	normal	normal	G2811A	A	G
h	normal	normal	G2811A	G2823T	G	G
i	normal	normal	C2810T	normal	G	G

Lane m is pBR322/MspI DNA size standard.

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Claims

1. A synthetic nucleotide construct for examining the human coagulation factor V gene, the synthetic construct being capable of forming a duplex with a fragment of the factor V gene, which fragment may contain the FV Leiden mutation, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene.
2. A synthetic nucleotide construct according to claim 1, wherein the 5 nucleotide deletion is within 10 nucleotides relative to the site of the possible mutation in the gene.
3. A synthetic nucleotide construct according to any claim 1 or 2, wherein the 5 nucleotide deletion comprises nucleotides -6 to -2 relative to the site of the possible mutation in the gene.
4. A synthetic nucleotide construct according to any preceding claim, wherein the synthetic construct is about 141 base pairs long.
5. A synthetic nucleotide construct according to any preceding claim, further comprising an inserted nucleotide.
6. A synthetic nucleotide construct according to claim 5, wherein the inserted nucleotide is positioned between nucleotides -7 and -8.

7. A synthetic nucleotide construct according to any preceding claim comprising [SEQ ID No:23]

8. A clone comprising the synthetic nucleotide construct according to any preceding claim.

5 9. A method of examining the factor V gene comprising the steps of:

a) forming fragments of the factor V gene, which fragments may contain the factor V Leiden mutation;

b) combining the fragments with a synthetic nucleotide
10 construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of possible mutation in the gene, and wherein the sequence of the synthetic construct is such that
15 there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene;

c) permitting duplex formation; and

d) separating the duplexes formed according to whether the mutation is present.

20 10. A method according to claim 9, wherein, prior to step (a), a patient's DNA and a sample containing the synthetic construct are mixed.

11. A method of forming a synthetic nucleotide construct for examining the human coagulation factor V gene, which may
25 contain the factor V Leiden mutation, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of a duplex, and wherein the sequence of the synthetic construct is such that

there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene, the method comprising the steps of:

- (a) generating a first fragment of the factor V gene using
5 a first and a second primer on either side of the part of the nucleotide sequence which may contain the mutation;
- (b) generating, from the gene or from the first fragment, a second fragment containing the 5 nucleotide deletion using the first or second primer and a third primer which contains
10 the deletion;
- (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it
15 to the end thereof; and
- (e) amplifying the extended strand.

12. A method according to claim 11, wherein the third primer is 5'-AAGAGCAGATCCCTGGCGAGGAATACAGGTA CTT-3' [SEQ ID No:1], the first primer is 5'-CATGAGAGACATCGCCTCTG-3' [SEQ
20 ID No:2], and/or the second primer is 5'-GACCTAACATGTTCTA-GCCAGAAG-3' [SEQ ID No:3].

13. A test kit for examining the human coagulation factor V gene, the kit comprising:

- (a) two primers suitable for use in PCR and capable of
25 annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence

under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the factor V Leiden mutation is present within the sequence under examination;
5 and

(c) a control DNA and/or control PCR amplification product, wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of the possible
10 mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is a 5 nucleotide deletion within 100 nucleotides relative to the site of the possible mutation in the gene.

14. A synthetic nucleotide construct for examining the
15 human methylenetetrahydrofolate reductase (MTHFR) gene, the synthetic construct being capable of forming a duplex with a fragment of the MTHFR gene, which fragment may contain a C to T mutation at nucleotide 677 in the coding sequence of the mRNA, wherein the nucleotide sequence of the synthetic
20 construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene.

25 15. A synthetic nucleotide construct according to claim 14, wherein the identifier is a 3 nucleotide deletion.

16. A synthetic nucleotide construct according to claim 15, wherein the identifier comprises nucleotides -4 to -2

relative to the site of the possible mutation in the gene.

17. A synthetic nucleotide construct according to any one of claims 14 to 16 comprising [SEQ ID No:25]

18. A clone comprising a synthetic nucleotide construct
5 according to any one of claims 14 to 17.

19. A method of examining the MTHFR gene comprising the steps of:

(a) forming fragments of the MTHFR gene, which fragments may contain a C to T mutation at nucleotide 677 in the
10 coding sequence of the mRNA;

(b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length
15 and spans the point of the possible mutation in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene;

(c) permitting duplex formation; and

20 (d) separating the duplexes formed according to whether the mutation is present.

20. A method according to claim 19, wherein, prior to step (a), a patient's DNA and a sample containing the synthetic construct are mixed.

25 21. A method of forming a synthetic construct for examining the human MTHFR gene which may contain a C to T mutation at nucleotide 677 in the coding sequence of mRNA, which construct is up to 500 base pairs in length and spans the

point of the possible mutation in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene, the method comprising the steps of:

- 5 (a) generating a first fragment of the MTHFR gene by a first and a second primer on either side of the part of the nucleotide sequence which may contain the mutation;
- (b) generating from the gene or from the first fragment, a second fragment containing the identifier using the first or
10 second primer and a third primer which contains the identifier;
- (c) mixing the first and second fragments in the presence of the first, second and third primers;
- (d) hybridising a strand of the second fragment to a
15 complementary strand of the first fragment and extending it to the end thereof; and
- (e) amplifying the extended strand.

22. A method according to claim 21, wherein the third primer is 5'-GAAGGAGAAGGTGTCTGCGGCCGATTTTCATCATCA-3' [SEQ ID
20 No:4], the first primer is 5'-AGGGAGCTTTGAGGCTGACCTGAA-3' [SEQ ID No:5], and/or the second primer is 5'-ACGATGGGGCAA-GTGATGCCCATG-3' [SEQ ID No:6].

23. A test kit for examining the human MTHFR gene, the kit comprising:

- 25 (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is

capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the C to T mutation at nucleotide 677 in the coding sequence of the mRNA is present within the sequence under examination; and

(c) a control DNA and/or control amplification product,

wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene.

24. A synthetic nucleotide construct for examining the gene encoding human prothrombin, the synthetic construct being capable of forming a duplex with a fragment of the gene encoding prothrombin, which fragment may contain a G to A mutation at nucleotide 20210 in the gene sequence, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene.

25. A synthetic nucleotide construct according to claim 24, wherein the identifier is a 3 nucleotide deletion.

26. A synthetic nucleotide construct according to claim 25, wherein the identifier comprises nucleotides +1 to +3 relative to the site of the possible mutation.

27. A synthetic nucleotide construct according to any one of claims 24 to 26, comprising 82 base pairs.

28. A synthetic nucleotide construct according to any one of claims 24 to 27 comprising [SEQ ID No:27]

29. A clone comprising a synthetic nucleotide construct according to any one of claims 24 to 28.

10 30. A method of examining the gene encoding human prothrombin comprising the steps of:

(a) forming fragments of the human prothrombin gene, which fragments may contain a G to A mutation at nucleotide 20210 in the gene;

15 (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene,
20 and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene;

(c) permitting duplex formation; and

(d) separating the duplexes formed according to whether the
25 mutation is present.

31. A method according to claim 30, wherein, prior to step (a), a patient's DNA and a sample containing the synthetic construct are mixed.

32. A method of forming a synthetic nucleotide construct for examining the gene encoding human prothrombin which may contain a G to A mutation at nucleotide 20210 in the gene sequence, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene, the method comprising the steps of:

(a) generating a first fragment of the gene encoding prothrombin using a first and second primer on either side of the part of the nucleotide sequence which may contain the mutation;

(b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;

(c) mixing the first and second fragments in the presence of the first, second and third primers;

(d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and

(e) amplifying the extended strand.

33. A method according to claim 32, wherein the third primer is 5'-GACTCTCAGCGCTCAATGCTC-3' [SEQ ID No:7], the first primer is 5'-GTGTTTCTAAACTATGGTTCCCAA-3' [SEQ ID No:8], and/or the second primer is 5'-CCCAGAGAGCTGCCCATGAATAG-3' [SEQ ID No:9].

34. A test kit for examining the gene encoding prothrombin,

the kit comprising:

- (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- 5 (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence
10 under examination dependent upon whether the G to A mutation at nucleotide 20210 is present within the sequence under examination; and
- (c) a control DNA and/or control PCR amplification product, wherein the synthetic nucleotide construct is up to 500
15 base pairs in length and spans the point of the possible mutation in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible mutation in the gene.
- 20 35. A synthetic nucleotide construct for examining the human coagulation factor 8 (F8) gene, the synthetic construct being capable of forming a duplex with a fragment of the F8 gene, which fragment may contain a T to A polymorphism in intron 18 of the F8 gene, wherein the
25 nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that

there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

36. A synthetic nucleotide construct according to claim 35, wherein the identifier is a 5 nucleotide deletion.

5 37. A synthetic nucleotide construct according to claim 36, wherein the identifier comprises nucleotides +2 to +6 relative to the site of the possible polymorphism in the gene.

38. A synthetic nucleotide construct according to claim 37,
10 wherein the identifier further comprises an insertion of a nucleotide.

39. A synthetic nucleotide construct according to claim 38, wherein the insertion is between nucleotides +9 and +10 relative to the site of the possible polymorphism in the
15 gene.

40. A synthetic nucleotide construct according to any one of claims 35 to 39 comprising [SEQ ID No:29]

41. A clone comprising a synthetic nucleotide construct according to any one of claims 35 to 40.

20 42. A method of examining the human coagulation F8 gene comprising the steps of:

(a) forming fragments of the human coagulation F8 gene, which fragments may contain a T to A polymorphism in intron 18 of the F8 gene;

25 (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming a duplex with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length

and spans the point of the possible polymorphism in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene;

- (c) permitting duplex formation; and
- (d) separating the duplexes formed according to whether the polymorphism is present.

43. A method according to claim 42, wherein, prior to step (a), a patient's DNA and a sample containing the synthetic construct are mixed.

44. A method of forming a synthetic nucleotide construct for examining the human coagulation F8 gene which may contain a T to A polymorphism in intron 18 of the F8 gene, which construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on formation of a duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene, the method comprising the steps of:

- (a) generating a first fragment of the human coagulation F8 gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain the polymorphism;
- (b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first or second primer and a third primer which contains the

identifier;

(c) mixing the first and second fragments in the presence of the first, second and third primers;

(d) hybridising a strand of the second fragment to a
5 complementary strand of the first fragment and extending it to the end thereof; and

(e) amplifying the extended strand.

45. A method according to claim 44, wherein the third primer is 5'-CAGTGATCAGATCAAGCTCCATGCTC-3' [SEQ ID No:10],
10 the first primer is 5'-TAAAAGCTTTAAATGGTCTAGGC-3' [SEQ ID No:11], and/or the second primer is 5'-TTCGAATTCTGAAATTATCTTGTTTC-3' [SEQ ID No:12].

46. A test kit for examining the human coagulation F8 gene, the kit comprising:

15 (a) two primers suitable for use in PCR and capable of annealing two complementary sequences at respective ends of a nucleotide sequence to be examined;

(b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence
20 under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether a T to A polymorphism in intron 18 of the F8 gene is present within
25 the sequence under examination; and

(c) a control DNA and/or control PCR amplification product, wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of the possible

mutation in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

5 47. A synthetic nucleotide construct for examining the human coagulation F8 gene, the synthetic construct being capable of forming a duplex with a fragment of the human coagulation F8 gene, which fragment may contain a G to A polymorphism in intron 7, wherein the nucleotide sequence of
10 the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible
15 polymorphism in the gene.

48. A synthetic nucleotide construct according to claim 47, wherein the identifier is an insertion of 3 nucleotides.

49. A synthetic nucleotide construct according to claim 48, wherein the insertion is positioned between nucleotides -1
20 and -2 relative to the site of the possible polymorphism in the gene.

50. A synthetic nucleotide construct according to any one of claims 47 to 49, wherein the identifier further comprises an insertion of a nucleotide between nucleotides +6 and +7
25 relative to the site of the possible polymorphism in the gene.

51. A synthetic nucleotide construct according to any one of claims 47 to 50 comprising [SEQ ID No:31] or [SEQ ID

No:32].

52. A clone comprising a synthetic nucleotide construct according to any one of claims 47 to 51.

53. A method of examining the human coagulation F8 gene comprising the steps of:

- (a) forming fragments of the F8 gene, which fragments may contain the G to A polymorphism in intron 7;
- (b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming
10 duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of the possible polymorphism in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides
15 relative to the site of the possible polymorphism in the gene;
- (c) permitting duplex formation; and
- (d) separating the duplexes formed according to whether the polymorphism is present.

20 54. A method of forming a synthetic nucleotide construct for examining the human coagulation F8 gene, which may contain a G to A polymorphism in intron 7, which construct is up to 500 base pairs in length and spans the point of the possible mutation in the gene on formation of a duplex, and
25 wherein there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene, the method comprising the steps of:

- (a) generating a first fragment of the F8 gene using a

first and a second primer on either side of the part of the nucleotide sequence which may contain the polymorphism;

(b) generating, from the gene or from the first fragment, a second fragment containing the identifier using the first
5 or second primer and a third primer which contains the identifier;

(c) mixing the first and second fragments in the presence of the first, second and third primers;

(d) hybridising a strand of the second fragment to a
10 complementary strand of the first fragment and extending it to the end thereof; and

(e) amplifying the extended strand.

55. A method according to claim 54, wherein the third primer is 5'-AGCAAGACACTCCCCTGACATTGCTTTGG-3' [SEQ ID
15 No:13], the first primer is 5'-AACAGCCTAATATAGCAAGACACTC-3' [SEQ ID No:14], and/or the second primer is 5'-CACATCCATTTTCAGAATCAGTAAG-3' [SEQ ID No:15].

56. A test kit for examining the human coagulation F8 gene, the kit comprising:

20 (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at the respective ends of a nucleotide sequence to be examined;

(b) a synthetic construct, which construct is capable of forming duplexes with the nucleotide sequence under con-
25 sideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether the G to A polymorphism

in intron 7 is present within the sequence under examination; and

(c) a control DNA and/or control amplification product,

wherein the synthetic nucleotide construct is up to 500
5 base pairs in length and spans the point of the possible polymorphism in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of the possible polymorphism in the gene.

10 57. A synthetic nucleotide construct for examining the human von Willebrand factor (VWF) gene, the synthetic construct being capable of forming a duplex with a fragment of the VWF gene, which fragment may contain one or more mutations including at least a G to A transition at position
15 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the coding sequence of the cDNA, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene
20 on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of one of the possible mutations in the gene.

58. A synthetic nucleotide construct according to claim 57,
25 wherein the identifier is positioned in a relative position between the site of the two possible mutations.

59. A synthetic nucleotide construct according to claim 57 or 58, wherein the identifier is a 3 nucleotide deletion.

60. A synthetic nucleotide construct according to any one of claims 57 to 59, further comprising an A to G transition and/or a T to G transversion.

61. A synthetic nucleotide construct according to any one
5 of claims 57 to 60 comprising [SEQ ID No:36] or [SEQ ID No:37]

62. A clone comprising a synthetic nucleotide construct according to any one of claims 57 to 61.

63. A method of examining the human von Willebrand factor
10 (VWF) gene comprising the steps of:

(a) forming fragments of the VWF gene, which fragments may contain one or more mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the
15 coding sequence of the cDNA;

(b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length
20 and spans the point of at least one of the possible mutations in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene;

25 (c) permitting duplex formation; and

(d) separating the duplexes formed.

64. A method of forming a synthetic nucleotide construct for examining the human VWF gene which may contain one or

more mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the coding sequence of the cDNA, which construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene, the method comprising the steps of:

- 10 (a) generating a first fragment of the VWF gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain at least one of the mutations;
- (b) generating, from the gene or from the first fragment, 15 a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;
- (c) mixing the first and second fragments in the presence of the first, second and third primers;
- 20 (d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and
- (e) amplifying the extended strand.

65. A method according to claim 64, wherein the third 25 primer is 5'-GTCGGGACCGGTGGAAGTGCAC-3' [SEQ ID No:16], the first primer is 5'-CAACTGTGCATCTCTGCCATGACTG-3' [SEQ ID No:17], and/or the second primer is 5'-CTCACCTGCACCAGAACGTACTGG-3' [SEQ ID No:18].

66. A test kit for examining the human VWF gene, the kit comprising:

- (a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of a nucleotide sequence to be examined;
- (b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are formed between the construct and the nucleotide sequence under examination dependent upon whether at least one of the mutations including at least a G to A transition at position 2811 in the coding sequence of the cDNA and/or a G to T transversion at position 2823 in the coding sequence of the cDNA is present; and

- (c) a control DNA and/or control PCR amplification product, wherein the synthetic nucleotide construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene.

67. A synthetic nucleotide construct for examining the human VWF gene, which fragment may contain one or more mutations including at least a C to T transition at position 2696 in the coding sequence of the cDNA and/or a T to A transversion at position 2701 in the coding sequence of the cDNA, wherein the nucleotide sequence of the synthetic

construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene on formation of the duplex, and wherein the sequence of the synthetic construct is such that there is an identifier
5 within 100 nucleotides relative to the site of at least one of the possible mutations in the gene.

68. A synthetic nucleotide construct according to claim 67, wherein the identifier is a 3 nucleotide insertion.

69. A synthetic nucleotide construct according to claim 68,
10 wherein the identifier is between nucleotides 0 and +1 relative to the site of the possible C to T transition.

70. A synthetic nucleotide construct according to any one of claims 67 to 69, wherein the identifier is in a relative position between the site of the two possible mutations.

15 71. A synthetic nucleotide construct according to any one of claims 67 to 70 comprising [SEQ ID No:34]

72. A clone comprising a synthetic nucleotide construct according to any one of claims 67 to 71.

73. A method of examining the human VWF gene, comprising
20 the steps of:

(a) forming fragments of the VWF gene, which fragments may contain one or more mutations including at least a C to T transition at position 2696 in the coding sequence of the cDNA and/or a T to A transversion at position 2701 in the
25 coding sequence of the cDNA;

(b) combining the fragments with a synthetic nucleotide construct, which synthetic construct is capable of forming duplexes with the fragments, wherein the nucleotide sequence

of the synthetic construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene, and wherein the sequence of the synthetic construct is such that there is an identifier within 100
5 nucleotides relative to the site of at least one of the possible mutations in the gene;

(c) permitting duplex formation; and

(d) separating the duplexes formed.

74. A method of forming a synthetic nucleotide construct
10 for examining the human VWF gene which may contain one or more mutations including at least a C to T transition at position 2696 in the coding sequence of the cDNA and/or a T to A transversion at position 2701 in the coding sequence of the cDNA, which construct is up to 500 base pairs in length
15 and spans the point of at least one of the possible mutations in the gene, and wherein there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene, the method comprising the steps of:

20 (a) generating a first fragment of the VWF gene using a first and a second primer on either side of the part of the nucleotide sequence which may contain at least one of the mutations;

(b) generating, from the gene or from the first fragment,
25 a second fragment containing the identifier using the first or second primer and a third primer which contains the identifier;

(c) mixing the first and second fragments in the presence

of the first, second and third primers;

(d) hybridising a strand of the second fragment to a complementary strand of the first fragment and extending it to the end thereof; and

5 (e) amplifying the extended strand.

75. A method according to claim 74, wherein the third primer is 5'-CTCTTAGGTCCAAAGGCATGAGAAC-3' [SEQ ID No:19], the first primer is 5'-AGGAGGGCTTTAGATCAGTCACTG-3' [SEQ ID No:20], and/or the second primer is 5'-
10 CAAGTGTTCAGCCAATCTTCACTG-3' [SEQ ID No:21].

76. A test kit for examining the human VWF gene, the kit comprising:

(a) two primers suitable for use in PCR and capable of annealing to complementary sequences at respective ends of
15 a nucleotide sequence to be examined;

(b) a synthetic nucleotide construct, which construct is capable of forming duplexes with the nucleotide sequence under consideration, the sequence of the construct being such that duplexes of different molecular conformation are
20 formed between the construct and the nucleotide sequence under examination dependent upon whether at least one of the mutations including at least a C to T transition at position 2696 and/or a T to A transversion at position 2701 in the coding sequence of the cDNA is present; and

25 (c) a control DNA and/or control PCR amplification product, wherein the nucleotide sequence of the synthetic construct is up to 500 base pairs in length and spans the point of at least one of the possible mutations in the gene,

and wherein the sequence of the synthetic construct is such that there is an identifier within 100 nucleotides relative to the site of at least one of the possible mutations in the gene.

5 77. A method of simultaneously examining a plurality of regions of genomic DNA comprising the steps of:

(a) forming a plurality of different fragments of the genomic DNA, which fragments may contain a known mutation or polymorphism, wherein each different fragment is formed by
10 a different set of primers;

(b) combining the fragments with a plurality of synthetic nucleotide constructs, each synthetic construct being capable of forming a duplex with a fragment of the genomic DNA;

15 (c) permitting duplex formation; and

(d) separating the different duplexes formed,
and wherein each set of primers function under the same conditions.

78. A method according to claim 77, wherein the factor V gene, MTHFR gene and the gene encoding prothrombin are
20 examined simultaneously.

79. A method according to claim 77, wherein different polymorphisms in the human F8 gene are examined simultaneously.

25 80. A method according to claim 77, wherein different mutations in the VWF gene are examined simultaneously.



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Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK CI (Ed.Q): G1B BAC

Int CI (Ed.6): C12Q 1/68

Other: ONLINE: WPI, EPODOC, CAS-ONLINE.

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	GB 2280266 A (UNIVERSITY OF BRISTOL) See whole document.	
A	Human Mutation 9(1) 1997 "Simultaneous Genotyping [...]" Jack, D. <i>et al.</i> pages 41-46.	
A	Br. J. Haematol. 97(3) 1997 "Genetic Detection of Factor V Leiden [...]" Bowen, D. <i>et al.</i> pages 961-692.	
X	Clinical Chemistry 42(3) 1996 "Heteroduplex Generator [...]" Stoerker, J. <i>et al.</i> pages 356-360. See especially page 357, Materials and Methods: "Criteria of importance..."	1, 8-11, 13, 14, 18-21, 23, 24, 29-32, 34, 35, 41-44, 46, 47, 52-54, 56, 57, 62-64 and 66

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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